Technical note: Copper chaperone for copper, zinc superoxide dismutase: A potential biomarker for copper status in cattle

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J Anim Sci 2009.87:4161-4166.
doi: 10.2527/jas.2009-1978 originally published online Aug 28, 2009;

Technical note: Copper chaperone for copper, zinc superoxide dismutase: A potential biomarker for copper status in cattle[1]

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ABSTRACT: Copper chaperone for Cu, Zn superoxide dismutase (CCS) has been shown to be reflective of Cu status in mice and rats. The objective of this study was to evaluate liver and erythrocyte CCS as an indicator of Cu status in beef cattle (Exp. 1), and to test the acute-phase properties of CCS under conditions of inflammation (Exp. 2). In Exp. 1, samples of whole blood and liver were collected at slaughter (492 d of age) from 15 Cu-deficient and 6 Cu-adequate Angus calves. At the time of tissue collection, severe Cu deficiency had been achieved and differences ($P < 0.0001$) in plasma and liver Cu among Cu-adequate and Cu-deficient calves were extreme (1.26 vs. 0.19 mg/L and 208.4 vs. 6.3 mg/kg for plasma and liver Cu, respectively). Protein levels of CCS were greater in liver (40%; $P = 0.02$) and erythrocytes (65%; $P < 0.0001$) of Cu-deficient vs. Cu-adequate calves. In Exp. 2, inflammatory responses were elicited in beef heifers by administration of a *Mannheimia hemolytica* vaccine. Four days after vaccination, plasma concentrations of the Cu-dependent protein ceruloplasmin and the Cu-independent protein haptoglobin were increased ($P < 0.001$) by 71 and 83%, respectively. In contrast, detection of CCS protein in samples of liver and erythrocytes did not differ ($P \geq 0.45$) between baseline (d 0) and d 4 after vaccination. These data demonstrate that bovine erythrocyte and liver CCS protein levels increase in Cu-deficient cattle. Furthermore, levels of CCS protein do not change after a vaccine-induced inflammatory response, suggesting that unlike ceruloplasmin, CCS may be a reliable indicator of Cu status in cattle.

Key words: acute-phase reaction, cattle, chaperone protein, copper

J. Anim. Sci. 2009. 87:4161–4166
doi:10.2527/jas.2009-1978

INTRODUCTION

Copper deficiency in cattle negatively affects multiple physiological functions related to growth, reproduction, and immune competence (McDowell, 2003; Underwood and Suttle, 1999). A reliable and practical method for assessing Cu status in live animals is currently not available. Assessment of liver Cu content is the most reliable indicator of Cu status, but collecting liver tissue from live cattle requires specialized training (Arthington and Corah, 1995) and may be impractical for on-farm cattle production environments. Blood Cu concentrations may be indicative of Cu status, but were shown to be influenced by fluctuating concentrations of ceruloplasmin, which represented as much as 95% of the total Cu found in blood (Cousins, 1985). Although concentrations of ceruloplasmin were shown to be dependent on the overall Cu status of cattle and reflective of the Cu content of the diet (Arthington et al., 1996), ceruloplasmin is an acute-phase protein whose concentrations increased after an inflammatory stimulus (Conner et al., 1988).

Copper chaperone for Cu, Zn superoxide dismutase (**CCS**) is essential for the delivery of Cu to, and subsequent activation of, the CCS apoenzyme (Wong et al., 2000). Protein levels of CCS have been shown to increase in the liver and erythrocytes of Cu-deficient mice and rats (Bertinato et al., 2003; West and Prohaska, 2004). To date, liver and erythrocyte CCS levels have not been characterized in the bovine. Therefore, the objectives of these experiments were 1) to determine if differences in bovine CCS levels could be detected in Cu-deficient vs. Cu-adequate beef cattle, and 2) to

[1]The authors thank A. Dunlap for technical assistance during the conduct of this study and Joseph Prohaska (Duluth School of Medicine, University of Minnesota, Duluth) for kindly providing us with a sample of anti-CCS antibody.

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Received March 23, 2009.
Accepted August 13, 2009.

determine if bovine CCS was responsive to an inflammatory challenge.

MATERIALS AND METHODS

The animal care, handling, and sampling procedures used in the study were approved by the North Carolina State University Animal Care and Use Committee.

Animals, Experimental Design, and Sample Collection and Processing

In Exp. 1, whole blood and samples of liver tissue were obtained from Angus calves (steers and heifers) enrolled in another study at North Carolina State University, Raleigh, designed to investigate the effects of high-Mn supplementation on measures of Cu status and growth in cattle (Hansen et al., 2009). Calves were derived from cows that had been enrolled in a previous study that examined Cu and Mn nutrition on the biology of brain prion proteins (Legleiter, 2006) and were approximately 500 d of age when tissue and blood samples were harvested. A complete description of diets, ingredients, and feeding methods has been published (Hansen et al., 2009). Samples were collected at the time of slaughter (n = 15 and 6 from Cu-deficient and Cu-adequate animals; mean age = 492 d), frozen at −80°C, and then shipped overnight on dry ice to the University of Florida, Gainesville. On arrival, samples were transferred to a freezer and stored frozen (−80°C) until later analysis. In Exp. 2, a total of 11 heifers (Brahman × British; mean age = 8.5 mo) were randomly selected from a larger group at the University of Florida Institute of Food and Agricultural Sciences, Range Cattle Research and Education Center, Ona. After selection, heifers were moved from perennial grass pastures (bahiagrass; *Paspalum notatum* Flueggé) into a single, partially shaded dry-lot pen (approximately 14 m^2) for 3 d of acclimation before vaccine administration. During this time, heifers were provided free-choice access to stargrass (*Cynodon* spp.) hay and 2.75 kg daily of an 80:20 blend of soybean hulls and cottonseed meal. At 0800 h on d 0, all heifers were administered a subcutaneous injection (2 mL) of *Mannheimia hemolytica* vaccine (One Shot, Pfizer Inc., New York, NY). Sampling occurred on d 0, immediately before vaccination, and continued daily at 0800 h for 4 consecutive days. On d 0 and 4, samples of liver were collected (via a liver biopsy technique; Arthington and Corah, 1995). Harvested liver samples were stored in a freezer at −80°C until later analysis for CCS. Samples of jugular blood (10 mL) were collected on d 0 (immediately before vaccine administration), 1, 2, 3, and 4 into Na heparin-coated (143 USP units) evacuated tubes (Vacutainer, Becton Dickinson Inc., Franklin Lakes, NJ) and immediately placed on ice. Each daily sample was transferred to 4°C and stored for 24 h before plasma was isolated by centrifugation at 2,000 × *g* for 20 min at room temperature. Plasma was stored frozen at −20°C until later analysis for ceruloplasmin and haptoglobin concentrations. Additional blood samples collected on d 0 and 4 were stored at 4°C for less than 24 h before erythrocyte processing by using a method modified from West and Prohaska (2004). Briefly, 1 mL of blood was centrifuged at 1,000 × *g* for 5 min at 4°C to pellet erythrocytes. The resulting supernatant was aspirated to remove the plasma and buffy coat, and the final red cell volume was recorded. Erythrocytes were resuspended by gently mixing in 1 mL of Alsever's solution (Sigma-Aldrich, St. Louis, MO). Cells were centrifuged and resuspended, as described above, a total of 3 times. After the final washing, a final red cell volume of approximately 200 μL remained. Cells were lysed by adding 200 μL of lysis buffer [10 m*M* Tris, pH 7.2, and 20 μL of 7× complete Mini Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN)]. Cell lysates were centrifuged at 13,000 × *g* for 10 min at 4°C to remove residual red cell debris. The supernatant was stored at −80°C until later analysis. Liver samples (0.5 to 0.85 g) were homogenized in 1 mL of lysis buffer and stored at −80°C until analysis.

Western Blot Analysis

Proteins were quantified using the RC DC Protein Assay (Bio-Rad, Hercules, CA). Proteins (80 μg) were mixed with Laemmli buffer and heated to 95°C for 5 min before loading onto a 12% polyacrylamide gel. Proteins were electrophoretically size-fractionated by SDS-PAGE and transferred to a 0.45-μm reinforced nitrocellulose membrane (Optitran BA-S 85, Whatman, Dassel, Germany). Nonspecific protein binding to the membrane was blocked by incubating the blot for 1 h in blocking buffer [5% nonfat dried milk suspended in Tris-buffered saline, pH 7.4, and 0.01% Tween-20 (**TBST**)]. A polyclonal antibody raised against full-length human CCS (Santa Cruz Biotechnology, Santa Cruz, CA) was used to detect bovine CCS, which shares 94% identity with the human protein. After primary antibody incubation for 1 h, the membranes were washed several times with TBST before incubation with the secondary antibody (horseradish peroxidase-linked donkey anti-rabbit IgG, GE Healthcare UK Limited, Little Chalfont Buckinghamshire, UK) for 40 min. Membranes were washed with TBST, followed by TBS (Tris-buffered saline, pH 7.4) before the final 5-min incubation with the chemiluminescence substrate (SuperSignal WestPico, Thermo Fisher Scientific, Waltham, MA). Blots were exposed to x-ray film for imaging. Antibodies were subsequently stripped by incubating the blot for 5 min at low pH (25 m*M* glycine, pH 2.8, 1% SDS). Blots were reprobed with rabbit anti-actin antibody (Sigma-Aldrich) or mouse anti-α-tubulin (clone B-5-1-2, Sigma-Aldrich) antibody as an indicator of protein loading. For the mouse primary antibody, a goat anti-mouse IgG horseradish peroxidase conjugate was used as a second antibody

in Western blotting. Immunoreactive band intensities were quantified by densitometric analysis (Gene Tools, SynGene, Fredrick, MD).

Plasma Ceruloplasmin and Haptoglobin Analyses

Plasma ceruloplasmin oxidase activity was measured in duplicate samples by using the colorimetric procedures described by Demetriou et al. (1974). The intraassay CV of duplicate samples was controlled to values of ≤10%. Ceruloplasmin concentrations were expressed as milligrams per deciliter, as described by King (1965). Interassay variation of both acute-phase protein assays were controlled by CV limits of ≤10%, as a result of a control sample analyzed in duplicate within each individual assay run. When the interassay CV exceeded 10%, all samples contained in the individual run with the control sample exceeding the average by the greatest were reanalyzed. This step was repeated until the results of standard pools for all runs resulted in a CV of ≤10%.

Plasma haptoglobin concentrations were determined in duplicate samples by measuring haptoglobin-hemoglobin complexing by the estimation of differences in peroxidase activity (Makimura and Suzuki, 1982). Results are expressed as arbitrary units resulting from the absorption reading × 100 at 450 nm. For samples with an absorption reading of ≤0.010, the intraassay CV of duplicate samples was controlled to values of ≤20%, and for samples with an absorption reading of ≥0.010, the intraassay CV of duplicate samples was controlled to values of ≤10%.

Statistical Analyses

Statistical analysis of variables for both experiments was achieved by ANOVA within a completely randomized design using the MIXED procedure (SAS Inst. Inc., Cary, NC). In Exp. 1, samples were obtained at the time of slaughter. Regardless of Cu status, calf sex had no impact on CCS levels in both liver and erythrocyte samples and thus was excluded from the final statistical model. The influence of Mn in the diet of Cu-deficient calves did not affect the magnitude of Cu deficiency achieved by the day of slaughter or the levels of CCS in either erythrocyte or liver samples. Therefore, all calves were considered either Cu-adequate or Cu-deficient, regardless of the presence of increased Mn in their diet. The final model statement contained the fixed effect of Cu status (Cu-adequate vs. Cu-deficient) and the random effect of animal (Cu status). In Exp. 2, the model statement contained the effect sampling time and the random effect of animal. Individual calf was the experimental unit for both experiments.

RESULTS

In Exp. 1, differences in liver and plasma Cu concentrations among Cu-deficient and Cu-adequate cattle were extreme ($P < 0.001$; 208.4 and 6.3 mg/kg of liver DM, and 1.26 and 0.19 mg/L of plasma; SEM = 7.94

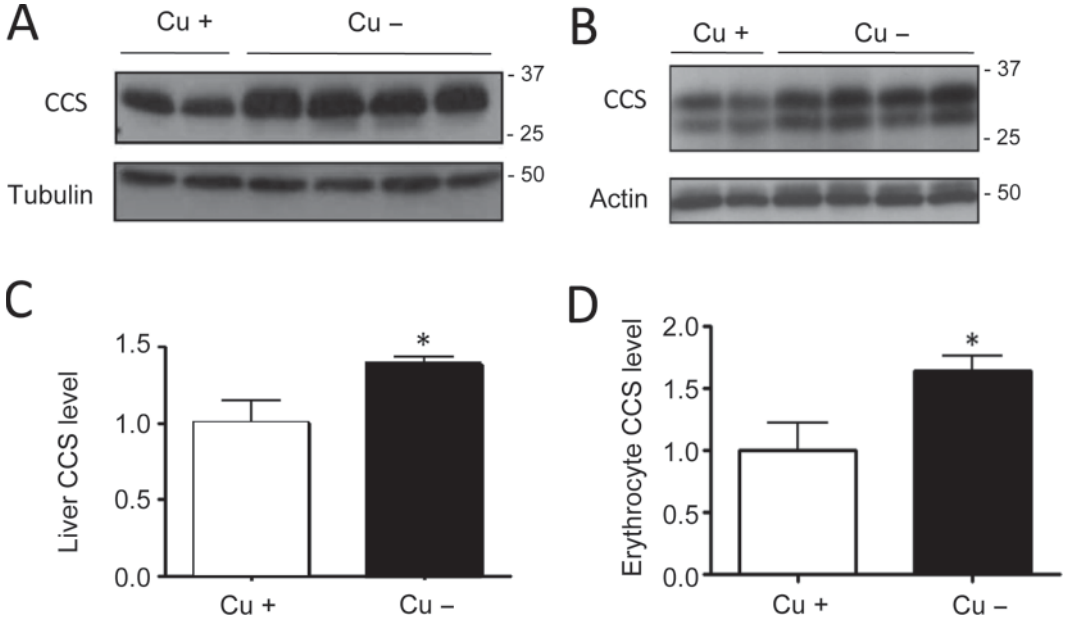

Figure 1. Bovine liver and erythrocyte Cu chaperone for Cu, Zn superoxide dismutase (CCS) levels increase with Cu deficiency. Proteins from liver (panel A) and erythrocyte (panel B) lysates were separated electrophoretically by SDS-PAGE, transferred to nitrocellulose, and analyzed for CCS by Western blot analysis. To visualize lane loading, the blot was stripped and reprobed for tubulin (A) or actin (B). Positions and masses of molecular weight markers (kDa) are shown to the right. Western blots show representative data from 2 Cu-sufficient (Cu+) and 4 Cu-deficient (Cu−) Angus cattle. Western blot CCS band intensities for all animals (Cu+, n = 6; Cu−, n = 15) were quantified by densitometry (panels C and D). Bars represent relative intensity/mean intensity (arbitrary units) of the Cu+ group ± SEM. *Differences ($P \leq 0.05$) among Cu status were observed for CCS expression in both liver and erythrocyte samples.

and 0.0652 for liver and plasma, respectively; Hansen et al., 2009). In Western blot analysis of bovine liver lysates, the antibody detected a single immunoreactive band that migrated with an apparent molecular mass of approximately 35 kDa (Figure 1A). The position of this immunoreactive protein was similar to that obtained from tissue samples from mouse (West and Prohaska, 2004), rat (Bertinato et al., 2003), and human blood analyzed in parallel with bovine liver (data not shown). Moreover, we detected the same immunoreactive band in bovine samples when we used a different anti-CCS antibody that was validated by analyzing samples from CCS-null mice (Prohaska et al., 2003; data not shown). Thus, it seems most probable that the anti-CCS antibody used for these studies detected the bovine CCS protein. In bovine erythrocytes, an additional band was detected at approximately 30 kDa (Figure 1B). Densitometric quantification of Western blot band intensities revealed a 40% increase ($P = 0.02$) in the levels of the 35-kDa CCS immunoreactive band in Cu-deficient bovine liver relative to Cu-adequate controls (Figure 1C). In erythrocytes, the corresponding CCS band increased by 65% ($P < 0.001$) in response to Cu deficiency (Figure 1D). Levels of the housekeeping proteins tubulin (Figure 1A) or actin (Figure 1B) did not change between groups, indicating equivalent protein loading among lanes.

In Exp. 2, all heifers were considered Cu-adequate, as determined by liver Cu concentrations (156 ± 55 mg/kg of liver DM). Administration of the *M. hemolytica* vaccine on d 0 induced an inflammatory response, as indicated by a 71 and 90% increase ($P < 0.001$) in plasma ceruloplasmin and haptoglobin concentrations by d 4 and 2 after vaccination, respectively (Figure 2). Western blot analysis of CCS protein in liver (Figure 3A) and erythrocyte (Figure 3B) samples and subsequent densitometric analysis (Figures 3C and D) demonstrated no changes from baseline (d 0) and 4 d after vaccination. The housekeeping proteins actin and tubulin also exhibited no changes in band intensity from d 0 to d 4 relative to vaccine administration (Figure 3A and B).

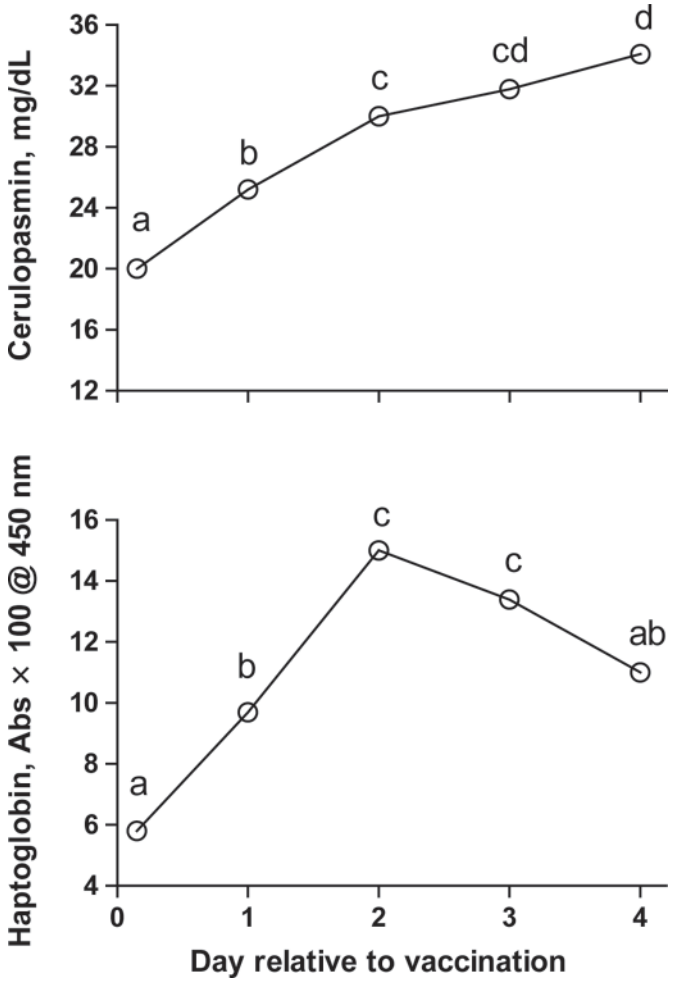

Figure 2. Plasma ceruloplasmin and haptoglobin concentrations in Brahman × British beef heifers (n = 11) administered *Mannheimia hemolytica* vaccine (d 0; One Shot, Pfizer Inc., New York, NY). Least squares means with unlike labels (a, b, c, and d) differ ($P < 0.05$). The SEM = 1.47 and 0.79 for ceruloplasmin and haptoglobin, respectively. Abs = spectrophotometer absorption reading.

DISCUSSION

A main finding of the present study was that Cu deficiency increased CCS levels in bovine liver and erythrocytes. Similar to the current study and working with samples collected from these same Angus calves, Hansen et al. (2009) reported an increase in CCS abundance in duodenal mucosal cells of Cu-deficient vs. Cu-adequate calves. These bovine research findings are similar to previous studies involving Cu-deficient rats and mice (Bertinato et al., 2003; West and Prohaska, 2004). The increase in CCS levels, however, was not as robust as that observed in the rodent models. Although this apparent discrepancy may reflect species-specific responses, it may also reflect differences in experimental designs. In rodent studies, weanling rats were fed Cu-deficient diets for approximately 30 d, whereas the calves in the current study were born to Cu-deficient dams and fed Cu-deficient diets for an average of 309 d (Hansen et al., 2009). Thus, calves were exposed to long-term Cu deficiency beginning from conception. Another difference between the bovine and rodent models relates to the concentrations of Cu in the liver. Although liver Cu concentrations were 30 times greater in the Cu-adequate relative to the Cu-deficient calves in the current study, the average concentration of liver Cu (6 ± 0.2 mg of Cu/kg of liver DM) was still 10 to 20 times greater than that in Cu-deficient rat liver (~0.3 to 0.6 mg of Cu/kg of liver DM). Indeed, Cu concentrations in Cu-deficient bovine liver are even greater than those in Cu-normal rat liver (3 to 4 mg of Cu/kg of liver DM; Prohaska et al., 2003; West and Prohaska, 2004). We therefore speculate that liver CCS levels increase more in rodents than in bovines because rodent tissue becomes substantially more depleted of Cu during deficiency. Alternatively, the long-term feeding of the Cu-deficient diet to the calves in the current study

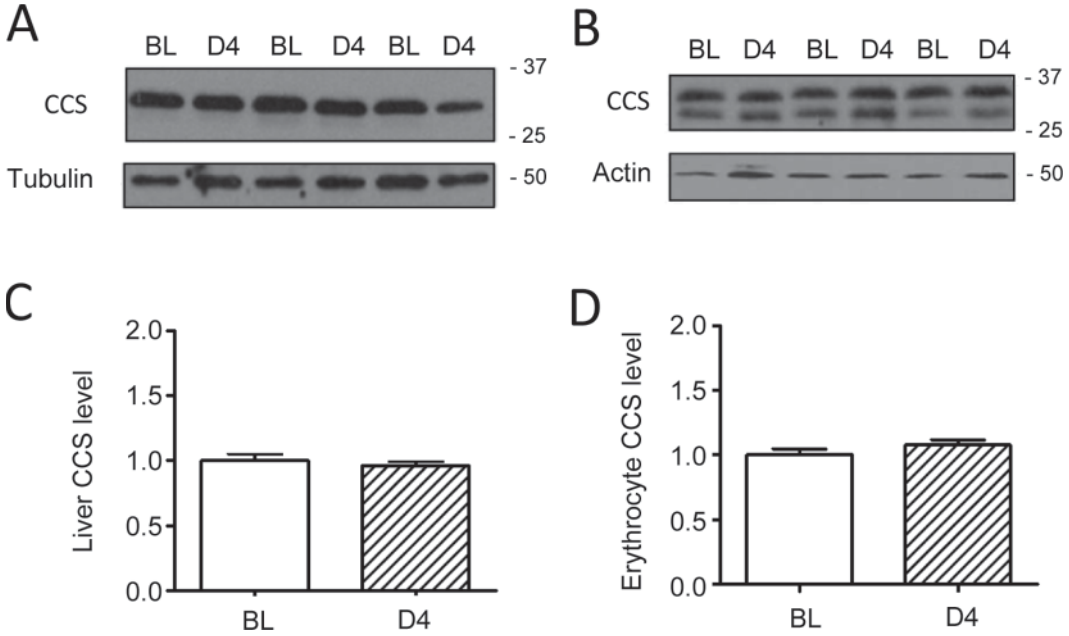

Figure 3. Bovine liver and erythrocyte Cu chaperone for Cu, Zn superoxide dismutase (CCS) levels do not change after an inflammatory challenge. Brahman × British beef heifers were administered a *Mannheimia hemolytica* vaccine (One Shot, Pfizer Inc., New York, NY). Samples of liver and erythrocytes were harvested at baseline (BL) and on d 4 after vaccination (D4). Proteins from liver (A) and erythrocyte (B) lysates were separated electrophoretically by SDS-PAGE, transferred to nitrocellulose, and analyzed for CCS by Western blot analysis. To visualize lane loading, the blot was stripped and reprobed for tubulin (A) or actin (B). Positions and masses of molecular weight markers (kDa) are shown to the right. Western blots show representative data at BL and D4 for 3 animals. Western blot CCS band intensities for all animals (n = 11) were quantified by densitometry (panels C and D). Bars represent relative intensity/mean intensity (arbitrary units) of BL values ± SEM.

may have provoked a compensatory response that prevented further depletion of tissue stores.

The results of Exp. 2 indicate that CCS protein levels do not change under conditions of inflammation. This finding is particularly important when considering the utility of using blood CCS levels as a potential indicator of Cu status in cattle. Inflammation is a major limitation for the use of serum or plasma as an indicator of Cu status because the majority of bloodborne Cu is bound to ceruloplasmin, which increases during inflammation. Plasma acute-phase proteins, nonspecific markers of inflammation (Heegaard et al., 2000; Arthington et al., 2003), were used in the present study to confirm the presence of an inflammatory response to the *M. hemolytica* vaccine. Increased plasma concentrations of ceruloplasmin, as well as other acute-phase proteins, occur rapidly (~24 h) during inflammatory conditions and after the initial proinflammatory cytokine stimulus (Baumann and Gauldie, 1994). In the present study, profiles of ceruloplasmin and haptoglobin, after vaccine administration, clearly indicated the presence of an acute-phase reaction; however, no changes in erythrocyte CCS were detected. This is likely because the protein amount is fixed once the red cell enucleates. However, to demonstrate that CCS responds only to chronic Cu deficiency and not to acute challenges, we measured it in red blood cells.

In these experiments, bovine erythrocyte and liver CCS was shown to be greater in Cu-deficient vs. Cu-adequate cattle; CCS thus holds potential as an indicator of Cu status in the bovine. Moreover, based on the findings of Exp. 2, CCS protein levels do not exhibit acute-phase properties, a limitation with current uses of blood Cu content as an indicator of Cu status. Additional studies are warranted to further evaluate CCS in cattle and to better determine the extent of its utility to serve as an indicator of Cu status.

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