Copper deficiency in the young bovine results in dramatic decreases in brain copper concentration but does not alter brain prion protein biology\textsuperscript{1,2}

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ABSTRACT: An Mn for Cu substitution on cellular prion proteins (PrP\textsuperscript{c}) in the brain that results in biochemical changes of PrP\textsuperscript{c} has been implicated in the pathogenesis of transmissible spongiform encephalopathies. Recent research in the mature bovine does not support this theory. The present study tested this hypothesis by using progeny from gestating cows receiving Cu-deficient diets or Cu-deficient diets coupled with high dietary Mn. Copper-adequate cows (n = 39) were assigned randomly to 1 of 3 treatments: 1) control (adequate in Cu and Mn), 2) Cu deficient (−Cu), or 3) Cu deficient plus high dietary Mn (−Cu+Mn). Cows assigned to treatments −Cu and −Cu+Mn received no supplemental Cu and were supplemented with Mo to further induce Cu deficiency. The −Cu+Mn treatment also received 500 mg of supplemental Mn/kg of dietary DM. Calves were weaned at 180 d and maintained on the same treatments as their respective dams for 260 d. Copper-deficient calves (−Cu and −Cu+Mn) had decreased (P = 0.001) brain (obex) Cu and tended to have increased (P = 0.09) obex Mn relative to control calves. Obex Mn:Cu ratios were substantially increased (P < 0.001) in calves receiving −Cu and −Cu+Mn treatments compared with control calves and were greater (P < 0.001) in −Cu+Mn calves than in −Cu calves. Obex prion protein characteristics, including protease K degradability, superoxide dismutase (SOD)-like activity, and glycoform distributions, were largely unaffected. Obex tissue antioxidant capacity was not compromised by perturbations in brain metals, but Cu-deficient calves tended to have decreased (P = 0.06) Cu:Zn SOD activity and increased (P = 0.06) Mn SOD activity. Although obex Cu was decreased because of Cu deficiency and Mn increased because of exposure to high dietary Mn, the obex metal imbalance had minimal effects on PrP\textsuperscript{c} functional characteristics in the calves.

Key words: bovine, copper, manganese, prion

INTRODUCTION

Bovine spongiform encephalopathy is one of several fatal neurodegenerative diseases in the family of transmissible spongiform encephalopathies (TSE) believed to be caused by the pathological isoform of the cellular prion protein (PrP\textsuperscript{c}; Prusiner, 1998). The mechanism(s) responsible for the conversion of PrP\textsuperscript{c} to the pathological isoform of PrP\textsuperscript{c} are not clear; however, the metal ions Cu and Mn have been implicated in the pathogenesis of TSE (Brown 2001; Lehmann, 2002). The binding of Cu ions (Kramer et al., 2001) may be important for prion structural stability (Hornshaw et al., 1995) and function (Brown et al., 1997; Brown, 1999; Wong et al., 2000). Manganese may also bind to PrP\textsuperscript{c} (Brown et al., 2000; Brown, 2001); however, a Mn for Cu substitution on PrP\textsuperscript{c} may impair PrP\textsuperscript{c} function (Lehmann, 2002; Deloncle et al., 2006) and allow for structural changes (Brown, 2001) that result in protease-resistant prions (Brown et al., 2000; Deloncle et al., 2006). Thus, an imbalance in brain Cu and Mn that allows Mn ions to replace Cu on PrP\textsuperscript{c} may be involved in the pathogenesis of prion diseases. This hypothesis is particularly attractive considering that large increases in brain Mn coupled with decreases in brain Cu have been associated with TSE (Wong et al., 2001a,b; Thackray et al., 2002).

Previous research conducted in this laboratory using the mature bovine (Legleiter et al., 2007a,b), as well as
that of other researchers using in vitro techniques and rodent models (Waggoner et al., 2000; Hutter et al., 2003; Jones et al., 2005), do not support this hypothesis. However, to our knowledge no research has been conducted to evaluate this hypothesis in young cattle. Thus, the objectives of this study were to determine the effects on brain Cu and Mn concentrations of exposing the young bovine to Cu deficiency or Cu deficiency plus high dietary Mn, and how variation of these metals may affect prion protein biochemical characteristics.

**MATERIALS AND METHODS**

**Animals and Experimental Design**

All care, handling, and sampling procedures were approved by the North Carolina State University Animal Care and Use Committee before the initiation of the experiment.

Thirty-nine Angus cows (5.6 ± 0.5 yr, 625 ± 19 kg) and their progeny were used in this study. Copper-adequate cows were assigned randomly (13 cows per treatment) to 1 of 3 treatments: 1) control (adequate in Cu and Mn), 2) Cu deficient (−Cu), and 3) Cu deficient plus high dietary Mn (−Cu+Mn). Supplemenal Cu was provided from CuCl₂ (Tri-Basic Copper Chloride, Micronutrients, Indianapolis, IN), Mn from MnSO₄·H₂O (Sulfamex, Veracruz, Mexico), and Mo from NaMoO₄ (Eastern Minerals Inc., Henderson, NC). To induce Cu deficiency, cows on treatments −Cu and −Cu+Mn were not supplemented with Cu and were provided 30 to 60 mg of Mo/d.

The goal was to induce Cu deficiency in gestating cows receiving treatments −Cu and −Cu+Mn, as well as to provide a high-Mn diet to treatment −Cu+Mn, during the third trimester of gestation such that the fetuses would be exposed to the imbalances in Cu and Mn in utero. The cows began receiving their respective treatments approximately 60 to 90 d prepartum and were maintained on those treatments until the calves were weaned at an average of 180 d. Cows grazed tall fescue pastures in treatment groups and were rotated systematically through pastures to minimize any pasteure effects. During the winter months, cows grazed stockpiled tall fescue and were supplemented with corn silage. The tall fescue pastures averaged 7.6 mg of Cu/kg of DM and 77.7 mg of Mn/kg of DM, and the corn silage averaged 5.9 mg of Cu/kg of DM and 44.4 mg of Mn/kg of DM. The Cu-deficient treatments received 75 mg of Mo/d in 0.9 kg of a corn supplement for the first 14 d to begin the depletion of Cu stores. For the first 120 d, the cows received their respective treatments through a free-choice mineral. The Cu-adequate treatment (control) contained 1,000 mg of Cu/kg of DM and 2,000 mg of Mn/kg of DM. Control cows consumed 130 mg supplemental Cu/d and 260 mg supplemental Mn/d, based on free-choice mineral consumption, which equates to 10.7 mg of Cu/kg of DM and 21.3 mg of Mn/kg of DM, assuming an average DMI of 2% of BW daily. These daily intakes of Cu and Mn are adequate to meet recommended requirements (NRC, 1996). The −Cu treatment contained 500 mg of Mo/kg of DM and 2,000 mg of Mn/kg of DM. Consumption by this treatment group averaged 240 mg of supplemental Mn/d or 20.0 mg of Mn/kg of DM, and 60 mg of supplemental Mo/d or 5.0 mg of Mo/kg of DM. The −Cu+Mn treatment contained 500 mg of Mo/kg of DM and 50,000 mg of Mn/kg of DM. Consumption by this treatment group averaged 6,000 mg of supplemental Mn/d or 500 mg of Mn/kg of DM, and 60 mg of supplemental Mo/d or 5.0 mg of Mo/kg of DM. From d 120 through weaning, all cows received a daily corn gluten feed supplement (1 kg/d) to provide the Mo (30 mg/d) to treatments −Cu and −Cu+Mn. Daily Mo supplementation more effectively depleted liver Cu stores compared with supplementing Mo via free-choice mineral. During this time, the cows continued to receive supplemental Cu (control) and Mn (all treatment groups) via the free-choice mineral. Liver biopsies were obtained from the cows, as described by Tiffany et al. (2003), 30 d prepartum and 60 and 180 d postpartum to determine Cu status. On the same days, jugular blood samples were collected into heparinized tubes (Vacutainer 9735, Becton Dickinson, Franklin Lakes, NJ) specifically designed for trace mineral analysis for plasma Cu determination. The volume of blood collected was 7 mL. Blood samples were placed on ice and later centrifuged at 1,200 × g for 20 min. Plasma was stored in acid-washed storage tubes and frozen at −20°C until analyzed for Cu.

Within 24 h of birth, the calves were weighed, a blood sample was collected for the determination of initial plasma Cu, and male calves were castrated. Blood samples were collected subsequently on d 60, 180, 350, and 440. Liver biopsies were taken on d 180 and 350.

At weaning (approximately 180 d of age), 30 of the calves (10 per treatment) were selected to continue on the study. Calves were selected based on dam Cu status, and attempts were made to balance each treatment group for sex. The steer and heifer calves were weighed and vaccinated against infectious bovine rhinotracheitis, bovine viral diarrhea (I and II), parainfluenza-3, bovine respiratory syncitial virus (Titanium 5, Agri-Labs, St. Joseph, MO),Clostridia (Vision 7, Intervet, Millsboro, DE), and *Moraxella bovis* (Piliguard Pink-eye-1 Trivalent, Schering-Plough Animal Health Ltd., Wellington, New Zealand). Cattle were also treated for internal and external parasites (Privermectin, First Priority Inc., Elgin, IL). They were housed in covered, slotted-floor pens with ad libitum access to fresh water. Calves were weaned and acclimated to Calan gates for 30 d before initiation of the growing phase. During the weaning and acclimation period, calves were fed corn silage and received no supplemental Cu, Mn, or Mo. Once acclimated, each calf began receiving the same treatment (except that supplemental Mo was reduced from 5 to 2 mg/kg of DM in the Cu-deficient diets) that their respective dam received during gestation and lactation. The treatments were formulated to provide the...
Table 1. Composition of the growing and finishing diets

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Growing</th>
<th>Finishing</th>
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<tr>
<td>Corn silage</td>
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<td>0.80</td>
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<td>Calcium sulfate</td>
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<td>0.40</td>
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<td>0.20</td>
</tr>
<tr>
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<td>0.01</td>
</tr>
<tr>
<td>Trace mineral premix2</td>
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<td>0.01</td>
</tr>
<tr>
<td>Monensin premix3</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>Treatment supplement4</td>
<td>2.00</td>
<td>2.00</td>
</tr>
</tbody>
</table>

1 Contained (per kg of premix): 6,600,000 IU of vitamin A; 1,520,000 IU of vitamin D; and 6,600 IU of vitamin E.
2 Contained (per kg of premix): 288 g of Zn as ZnSO₄; 4.8 g of I as Ca(IO₃)₂(H₂O); 1.9 g of Se as Na₂SeO₃; and 1.0 g of Co as CoCO₃.
3 Provided 33 mg of monensin/kg of dietary DM.
4 A supplement based on ground corn provided the following treatments: control (10 mg of Cu/kg of DM, 20 mg of Mn/kg of DM); −Cu (20 mg of Mn/kg of DM, 2 mg of Mo/kg of DM); −Cu+Mn (500 mg of Mn/kg of DM, 2 mg of Mo/kg of DM).

following (supplemental concentrations expressed per kilogram of dietary DM): control (10 mg of Cu/kg of DM, 20 mg of Mn/kg of DM); −Cu (20 mg of Mn/kg of DM, 2 mg of Mo/kg of DM); and −Cu+Mn (500 mg of Mn/kg of DM, 2 mg of Mo/kg of DM). The calves received a corn silage-based growing diet (Table 1; 7.0 mg of Cu/kg of DM, 42.7 mg of Mn/kg of DM) once daily in amounts adequate to allow ad libitum intake, with their corresponding treatment provided in a corn supplement at 2% of dietary DM. After feeding the growing diet for 140 d, the steers and heifers were implanted with Synovex-Plus (Fort Dodge Animal Health, Fort Dodge, IA) and switched gradually (over a 14-d period) to a corn-based finishing diet (Table 1; 7.0 mg of Cu/kg of DM, 17.6 mg of Mn/kg of DM) for 120 d. All diets were formulated to meet or exceed all nutrient requirements except for Cu (NRC, 1996). Cattle were fed individually and were weighed every 28 d.

At approximately 470 d of age, the steers and heifers were transported approximately 320 km to an abattoir and slaughtered after an overnight period of feed withdrawal. A liver sample (approximately 100 g) was collected, transported on dry ice, and stored frozen (−20°C) until analysis. The obex portion of the brain stem (between the cerebellum and spinal cord, which contains the motor nucleus of the vagus nerve) was removed through the occipital foramen by using the spoon technique (Legleiter et al., 2007b). The obex was transported on dry ice and stored (−80°C) until analysis.

Analytical Procedures

Liver, obex, and feed samples were prepared for Cu and Mn analysis by using the microwave digestion (Mars 5, CEM Corp., Matthews, NC) procedure described by Gengelbach et al. (1994). Before microwave digestion, approximately 0.3 g of dried tissue or 0.5 g of dried feed was allowed to digest overnight in trace mineral-grade nitric acid (Fisher Scientific, Fair Lawn, NJ). Tissue and feed Cu and Mn were determined by acetylene flame atomic absorption spectrophotometry (GFA-6500, Shimadzu Scientific Instruments, Kyoto, Japan).

Plasma Cu was determined as described by Legleiter and Spears (2007). Briefly, plasma was diluted 1:3 (vol/vol) in 5% trace mineral-grade nitric acid (Fisher Scientific), centrifuged at 1,200 × g for 20 min, and analyzed for Cu by using acetylene flame atomic absorption spectrophotometry (GFA-6500, Shimadzu Scientific Instruments).

Total protein was extracted from obex tissue according to the procedure of Wong et al. (2000) as described previously (Legleiter et al., 2007a,b). Briefly, chilled obex tissue was homogenized on ice in extraction buffer and centrifuged immediately. The clarified supernatant was analyzed for total protein by using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA) so that the samples could be equilibrated based on protein concentration. The protein-equilibrated supernatants were aliquotted into microcentrifuge tubes and stored at −80°C until analysis.

All electrophoresis and Western blot (WB) procedures have been described previously (Legleiter et al., 2007a,b). Polyacrylamide gel electrophoresis (Novex X-Cell Surelock Mini-Cell, Invitrogen Corp., Carlsbad, CA) was performed by using precast gels (NuPAGE Novex 10% Bis-Tris, Invitrogen Corp.). Molecular weight standards (Magic Mark XP Western Protein Standard, Invitrogen Corp.) were used for molecular weight (MW) estimation, and recombinant PrP (ab753, Abcam Inc., Cambridge, MA) served as a positive control. After transfer to a polyvinylidene fluoride membrane, the WB was visualized (Western Breeze Chemiluminescent Kit, Invitrogen Corp.). Immunoreactive PrP were probed with anti-PrP (mAb 6H4, Prionics AG, Zurich, Switzerland), and the WB image was captured on film (Kodak X-OMAT LS, Eastman Kodak Co., Rochester, NY) and analyzed (Image Quant TL, Amersham Biosciences, Piscataway, NJ). Analysis included band identification, MW estimation based on standardized MW markers, and relative optical densitometry for each glycoform of PrP. All WB analyses were replicated 2 to 3 times.

To determine the effects of treatment on prion proteinase degradability, samples were first exposed to proteinase K (PK; Bio-Rad Laboratories Inc.) as described by Thackray et al. (2002) before PAGE and WB. Briefly, 10% obex tissue homogenates were exposed to 250 μg of PK/mL for 1 h at 37°C. Both unexposed and PK-exposed samples for each animal were run parallel to one another on the same gel. The PK protocol described by Brown et al. (2000), using 0, 2, 10, and 25 μg of PK/mL of 10% obex tissue homogenate, was used to more sensitively test the effects of treatment on
the proteinase degradability of prion proteins. For both PK tests, the reaction was stopped with the addition of loading buffer, reducing agent, and heating to 70°C. Proteinase degradability was determined by comparing the WB elution profiles of PrP<sup>c</sup> exposed to PK with those not exposed to PK. Complete PK degradation resulted in no detectable immunoreactive prion proteins in PK-treated lanes on the WB.

A double-antibody sandwich ELISA (Cayman Chemical Co., Ann Arbor, MI) was used to quantify PrP<sup>c</sup> in PK-treated lanes on the WB. The resulting ferric ions were detected by their ability to oxidize ferrous ions. The resulting ferric ions were quantified by using thiocyanate as a chromagen, and were monitored by measuring absorbance at 500 nm.

Prion proteins from all obex tissue homogenates were purified by using immunoprecipitation, based on the methods described by Brown et al. (1999). The PrP<sup>c</sup> monoclonal antibody 6H4 was coupled to protein G-agarose (Sigma-Aldrich Inc., St. Louis, MO) and subsequently mixed with obex tissue homogenates overnight at 4°C in microtube spin columns. The agarose beads were washed extensively and proteins were subsequently eluted from the beads with the addition of 50 mM glycine (pH 4.0), followed by neutralization of the eluate with 100 mM Tris-HCl (pH 8.0). The protein concentrations of the purified PrP<sup>c</sup> eluates were determined colorimetrically in a 96-well format. Similar to the Lowry assay, color development was achieved through a 2-step process, including the reaction between protein and copper tartrate in an alkaline solution and the subsequent reduction of Folin reagent by the Cu-treated protein. Absorbance was measured at 750 nm, with protein concentrations ranging from 200 μg/mL to 1.0 mg/mL (Bio-Rad DC, Bio-Rad Laboratories Inc.). The purity of the immunopurified prion eluates was confirmed (>90%) by elution on polyacrylamide gels, followed by Coomassie blue staining, and the presence of PrP<sup>c</sup> was confirmed by immunodetection on nitrocellulose membranes (i.e., dot blotting). The SOD-like activity of immunopurified PrP<sup>c</sup> was determined as described for obex tissue homogenates. Immunopurified PrP<sup>c</sup> eluates were also analyzed for Cu and Mn concentrations by using electrothermal (graphite furnace) atomic absorption spectrophotometry (AA-6701F with GFA-6500, Shimadzu Scientific Instruments).

**Statistical Analysis**

All data was analyzed by using PROC MIXED (SAS Inst. Inc., Cary, NC). Plasma Cu and liver Cu and Mn values collected over the duration of the study were analyzed as repeated measures, with fixed effects of treatment, time, sex, and all interactions. Animal served as a random effect and the covariance structure was spatial power. Interactions that were not significant (P > 0.05) were removed from the model. All performance data and postmortem obex, liver, and prion data were analyzed as a completely randomized design, with fixed effects of treatment, sex, and treatment × sex. Animal served as the experimental unit. When the treatment × sex interaction was not significant (P > 0.05), it was removed from the model and sex served as a random effect. Gel was also included in the model as a fixed effect when WB data were analyzed. For all analyses, treatment means were separated by using 2 preplanned single-df orthogonal contrasts: 1) control vs. −Cu and −Cu+Mn; and 2) −Cu vs. −Cu+Mn. Effects were considered significant at P < 0.05. During the growing phase, 2 calves died (1 each from treatments −Cu and −Cu+Mn).
RESULTS

Data pertaining to the dams has previously been reported (Legleiter et al., 2007b). Briefly, 30 d before calving, liver Cu concentrations tended ($P = 0.07$) to be decreased in −Cu (123.5 mg of Cu/kg of DM) and −Cu+Mn cows (105.8 mg of Cu/kg of DM) compared with control cows (174.9 mg of Cu/kg of DM). However, cows receiving treatments −Cu and −Cu+Mn were not Cu deficient before calving, based on liver Cu concentrations. Providing supplemental Mo in the free-choice mineral proved to be ineffective in rapidly inducing a Cu deficiency. Once the cows assigned to treatments −Cu and −Cu+Mn began receiving Mo in a daily supple-

ment, they subsequently reached a Cu-deficient status between d 60 and 180 postpartum. Liver Cu concentrations on d 180 averaged 21.0 and 19.6 mg of Cu/kg of DM for the −Cu and −Cu+Mn treatments, respectively, compared with 214.8 mg of Cu/kg of DM for the control treatment (Legleiter et al., 2007b).

All calves appeared to be healthy at birth, but calves from dams receiving treatment −Cu+Mn were lighter ($P = 0.03$), at 37.3 kg, than those from dams receiving treatment −Cu, at 41.7 kg. Weaning BW were not different across treatments, with an overall average of 240.2 kg. Performance data from the growing and finishing phases have been reported previously (Legleiter and Spears, 2007).

When analyzed as repeated measures, treatment ($P < 0.001$), day ($P < 0.001$), and the interaction of treatment × day ($P < 0.001$) affected plasma Cu concentrations in the calves (Figure 1). Both treatments designed to induce Cu deficiency (−Cu and −Cu+Mn) resulted in less plasma Cu concentrations ($P \leq 0.02$) on d 0, 180, 350, and 440, but not on d 90. Plasma Cu was further depressed in animals receiving −Cu+Mn compared with those receiving −Cu on d 350 ($P = 0.10$) and d 440 ($P < 0.001$).

Liver Cu concentrations in calves were affected by treatment ($P < 0.001$), day ($P = 0.01$), and an interaction of treatment × day ($P < 0.001$) when analyzed as repeated measures. Treatments −Cu and −Cu+Mn resulted in less ($P < 0.001$) liver Cu concentrations compared with control animals on d 180, 350, and 440 (Figure 2). Final liver Cu concentrations from slaughtered calves were reduced ($P = 0.001$) in treatments −Cu and −Cu+Mn relative to the control group (Table 2). Both treatments designed to induce Cu deficiency (−Cu and −Cu+Mn) resulted in liver Cu concentrations of less than 20 mg/kg of DM and would therefore be considered Cu deficient (Underwood, 1981).

Liver Mn concentrations were affected by treatment ($P < 0.001$), day ($P < 0.001$), and treatment × day ($P = 0.03$). Cattle receiving high levels of Mn (−Cu+Mn) had greater ($P < 0.001$) liver Mn concentrations on d 350 and 440, but not d 180, compared with cattle receiving treatment −Cu (Figure 3). Within the Cu-deficient treatments, ending liver Mn was greater ($P = 0.001$) in cattle receiving treatment −Cu+Mn (Table 2).

To further illustrate the imbalance in Cu and Mn, ratios of Mn:Cu were compared between treatments. Liver Mn:Cu ratios were greater ($P < 0.001$) in calves receiving the −Cu and −Cu+Mn treatments compared with the control treatment and were greater ($P < 0.001$) in −Cu+Mn calves than in −Cu calves (Table 2).

Obex Cu and Mn concentrations followed patterns similar to liver Cu and Mn concentrations. Obex Cu concentration was decreased ($P = 0.001$) in the Cu-deficient treatments compared with control cattle (Table 2). Obex Mn concentration was greater ($P = 0.001$) in cattle receiving treatment −Cu+Mn compared with treatment −Cu. Likewise, obex Mn:Cu ratios were greater ($P < 0.001$) in calves receiving the −Cu and −Cu+Mn
treatments compared with the control treatment and were greater \((P < 0.001)\) in −Cu+Mn calves than in −Cu calves (Table 2).

Densitometric analysis of WB indicated that \(\text{PrP}^c\) band relative optical densities were similar \((P \geq 0.57)\) across all treatments, with −Cu and −Cu+Mn averaging 99.7 and 101.3 ± 2.1%, respectively, of the control treatment. Obex prion protein concentrations, as determined by ELISA, were also similar \((P = 0.24)\) among treatments (Table 3). Thus, based on both ELISA-quantified and WB relative optical densities, \(\text{PrP}^c\) concentrations were not affected by obex Cu and Mn concentrations.

The estimated MW of the \(\text{PrP}^c\) glycoforms were similar \((P \geq 0.34)\) across the 3 treatments, averaging 35.8, 32.3, and 27.1 ± 0.15 kDa for di-, mono-, and unglycosylated \(\text{PrP}^c\), respectively. Likewise, the relative distribution of the 3 glycoforms was not affected by treatment \((P \geq 0.14)\), nor did there appear to be any apparent differences in the banding patterns of the \(\text{PrP}^c\) from all obex tissue homogenates (Figure 4).

All prion proteins were degraded completely, as determined by WB analysis, when exposed to 10 and 25 \(\mu\)g of PK/mL of 10% obex tissue homogenate (Figure 5). However, 2 \(\mu\)g of PK/mL of 10% obex tissue homogenate allowed for some \(\text{PrP}^c\) to remain intact and visible on the WB. This visible band was present in all samples, likely because of a very low and less than saturable PK enzyme concentration. The PK test indicates that treatment did not affect \(\text{PrP}^c\) proteinase degradability and that animals were bovine spongiform encephalopathy negative.

Obex tissue total SOD activity tended to be less \((P = 0.08)\) in the −Cu treatment compared with the −Cu+Mn treatment (Table 3). The Cu:Zn SOD activity tended \((P = 0.06)\) to be less in the Cu-deficient treatments compared with the control treatment. Alternatively, Mn SOD activity tended \((P = 0.08)\) to be increased in the Cu-deficient treatments compared with the control treatment. The overall antioxidant capacity of obex tissue homogenates was similar \((P \geq 0.39)\) across all treatments, averaging 0.14, 0.15, and 0.16 ± 0.02 Trolox equivalents for the control, −Cu, and −Cu+Mn treatment, respectively, indicating that obex metal perturbations did not compromise the antioxidant defense systems. Lipid hydroperoxides, resulting from lipid peroxidation, were assayed in obex tissue homogenates.

### Table 2. Effect of dietary Cu and Mn content on liver and obex Cu and Mn concentrations

<table>
<thead>
<tr>
<th>Item</th>
<th>Control</th>
<th>−Cu</th>
<th>−Cu+Mn</th>
<th>SEM</th>
<th>Treatment vs. −Cu and −Cu+Mn</th>
<th>SEM</th>
<th>−Cu vs. −Cu+Mn</th>
<th>SEM</th>
</tr>
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<td>Cu, mg/kg of DM</td>
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<td></td>
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<tr>
<td>Liver</td>
<td>161.5</td>
<td>5.9</td>
<td>4.2</td>
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<tr>
<td>Obex</td>
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<td>Mn, mg/kg of DM</td>
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<td>Liver</td>
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<td>0.06</td>
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</table>

1Samples collected immediately after slaughter.
2Treatments: 1) control (adequate in Cu and Mn), 2) Cu deficient (−Cu), and 3) Cu deficient plus high dietary Mn (−Cu+Mn).
3P-values for preplanned single-df contrasts.

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Figure 2. Average liver Cu stores for each treatment from d 180 to 470. Analyzing the data as repeated measures by using PROC MIXED (SAS Inst. Inc., Cary, NC) revealed the main effects of treatment \((P < 0.001)\), day \((P = 0.01)\), and an interaction of treatment × day \((P < 0.001)\). The interaction was further defined by analyzing the effect of treatment for each day by using PROC MIXED in SAS and single-df contrasts to separate the means. Both treatments designed to induce Cu deficiency (−Cu and −Cu+Mn) resulted in reduced liver Cu concentrations \(* control vs. −Cu and −Cu+Mn, P < 0.001\) compared with control animals on d 180, 350, and 440. Treatments: 1) control (adequate in Cu and Mn), 2) Cu deficient (−Cu), and 3) Cu deficient plus high dietary Mn (−Cu+Mn).
Immunopurified PrP c had similar ($P \geq 0.16$) SOD-like activity across treatments and did not differ ($P \geq 0.24$) in Cu concentration (Table 3). Manganese was detectable at low concentrations (<1 μg/g) in some treatments, but all were below the detection limit (0.25 nmol of hydroperoxide) of the assay (data not shown).

### Figure 3

Average liver Mn concentrations for each treatment from d 180 to 470. Analyzing the data as repeated measures by using PROC MIXED (SAS Inst. Inc., Cary, NC) revealed the main effects of treatment ($P < 0.001$), day ($P < 0.001$), and an interaction of treatment × day ($P = 0.03$). The interaction was further defined by analyzing the effect of treatment for each day by using PROC MIXED in SAS and single-df contrasts to separate the means. Within the Cu-deficient treatments (−Cu and −Cu+Mn), cattle receiving high levels of Mn (−Cu+Mn) had greater liver Mn concentrations ($−Cu$ vs. $−Cu+Mn$, $P < 0.001$) compared with $−Cu$ animals on d 350 and 440. Treatments: 1) control (adequate in Cu and Mn), 2) Cu deficient (−Cu), and 3) Cu deficient plus high dietary Mn (−Cu+Mn).

### Table 3

Effect of dietary Cu and Mn on obex homogenate superoxide dismutase (SOD) activities and prion protein concentration (PrP) and immunopurified prion protein SOD activity and Cu concentration

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment $^2$</th>
<th>SEM</th>
<th>Contrast $^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>−Cu</td>
<td>−Cu+Mn</td>
</tr>
<tr>
<td>Obex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total SOD, $^4$ U/mg</td>
<td>71.2</td>
<td>63.0</td>
<td>72.4</td>
</tr>
<tr>
<td>Cu/Zn SOD, U/mg</td>
<td>56.2</td>
<td>45.3</td>
<td>49.6</td>
</tr>
<tr>
<td>Mn SOD, U/mg</td>
<td>14.9</td>
<td>17.8</td>
<td>22.9</td>
</tr>
<tr>
<td>PrP$^5$, ng/g</td>
<td>1.14</td>
<td>1.00</td>
<td>1.04</td>
</tr>
<tr>
<td>Prion protein SOD, $^6$ U/mg</td>
<td>63.7</td>
<td>76.2</td>
<td>77.5</td>
</tr>
<tr>
<td>Cu, $^7$ mg/g</td>
<td>0.43</td>
<td>0.63</td>
<td>0.29</td>
</tr>
</tbody>
</table>

$^1$All assays performed in duplicate or triplicate.

$^2$Treatments: 1) control (adequate in Cu and Mn), 2) Cu deficient (−Cu), and 3) Cu deficient plus high dietary Mn (−Cu+Mn).

$^3$P-values for preplanned single-df contrast.

$^4$Expressed as U/mg of protein in the 10% obex tissue homogenate, where 1 U = activity required for 50% dismutation of the superoxide radicals.

$^5$Expressed as ng of cellular PrP (PrP)/g of obex tissue.

$^6$Expressed as U/mg of protein in the immunopurified PrP$^5$ eluate.

$^7$Expressed as mg of Cu/g of protein in the immunopurified PrP$^5$ eluate.

### Figure 4

Effects of dietary Cu and Mn levels on prion protein elution profiles. The Western blots (WB) are of prion proteins (panels A, B) from obex tissue homogenates from all 28 steers and heifers. Visual analysis of the WB shows similar prion protein banding patterns across treatments. Treatments: 1) Cu adequate (control); 2) Cu deficient (−Cu); 3) Cu deficient plus high dietary Mn (−Cu+Mn). β-Actin was used to normalize all lanes (panels a, b).
munopurified PrPc eluates, but most samples had undetectable concentrations of Mn (data not shown).

**DISCUSSION**

Our initial objective of inducing of Cu deficiency in newborn calves was unsuccessful because the dams were not deficient before parturition, and calves showed no signs of Cu deficiency at birth. However, the calves in treatments −Cu and −Cu+Mn did become Cu deficient at an early age. According to liver Cu concentrations, the calves assigned to treatments −Cu and −Cu+Mn were deficient at weaning (180 d). However, because liver biopsies were not performed before weaning, it was not clear when liver Cu concentrations were depleted below 20 mg of Cu/kg of DM. Plasma Cu concentrations in treatments −Cu and −Cu+Mn were also less at weaning relative to the control concentration, but were above the 0.5 μg/mL threshold that is indicative of Cu deficiency (Underwood, 1981). However, it has been reported previously that plasma Cu values may indicate cattle are Cu adequate, whereas liver Cu concentrations are indicative of Cu deficiency (Mullis et al., 2003; Ahola et al., 2005). Thus, it is not possible to identify the precise age at which −Cu and −Cu+Mn calves became Cu deficient. Nonetheless, combining the liver Cu and plasma Cu data suggests that calves were Cu deficient throughout at least most of the growing phase and all of the finishing phase.

Calf liver Mn concentrations at weaning were similar for all treatments but rapidly increased in calves receiving treatment −Cu+Mn from weaning through the end of the study. The increase, from approximately 10 to 16 mg of Mn/kg of DM, was similar to that seen in cattle supplemented with 240 mg of Mn/kg of DM vs. 10 mg of supplemental Mn/kg of DM (Legleiter et al., 2005). Conversely, liver Mn concentrations were not affected in mature cows receiving similar levels of Mn in a free-choice mineral (Legleiter et al., 2007b). The discrepancy between studies using mature cows and growing calves may be related to age or route of Mn administration (diet vs. free-choice mineral).

The treatments −Cu and −Cu+Mn were effective in altering the Cu and Mn status of the calves, which subsequently changed the oxob Cu and Mn profiles. Oxob Cu concentrations were decreased by 60 and 72% for treatments −Cu and −Cu+Mn, respectively. Additionally, exposure to 500 mg of Mn/kg of DM in treatment −Cu+Mn resulted in a 31% increase in oxob Mn concentration compared with cattle receiving 20 mg of Mn/kg of DM (−Cu). Further, the substantial differences in the Mn:Cu ratios emphasize the magnitude of the imbalances in Cu and Mn induced by the −Cu and −Cu+Mn treatments. These induced perturbations in both oxob Cu and Mn concentrations were more pronounced than in previous studies using mature cows (Legleiter et al., 2007a,b); thus, as expected, young calves appear to be more susceptible to changes in oxob metal concentrations than mature cows.

The perturbed oxob Cu and Mn concentrations tended to alter the SOD activities of the oxob. Specifically, Mn SOD activity in Cu-deficient animals tended to be increased compared with controls. However, unlike in mature cows (Legleiter et al., 2007b), there was a concomitant decrease in Cu:Zn SOD activity in the Cu-deficient calves. The decrease in Cu:Zn SOD activity in −Cu+Mn calves appears to have been offset by the increased Mn SOD; however, this was not the case for −Cu calves, thus explaining the tendency for decreased total SOD activity. Although these differences are only statistical trends, the oxob tissue SOD activities were apparently affected to a greater extent by Cu deficiency in these calves than in the mature cows described previously (Legleiter et al., 2007b). This is likely a reflection of the more dramatically affected oxob Cu and Mn concentrations in the younger calves compared with the mature cows. However, realizing no changes in the total antioxidant capacity of the oxob tissue coupled with the lack of detectable lipid hydroperoxides indicates that the altered oxob Cu and Mn concentrations and subsequent effects on the SOD activities did not increase oxidative stress.
As reported previously (Legleiter et al., 2007a,b), a decrease in obex Cu concentration, alone or coupled with an increase in Mn concentration, had minimal effects on the biochemical properties of PrPc. Most notably, there were no detectable changes in immunopurified PrPc Cu and Mn concentrations, SOD-like activity, or PK degradability. This is in contrast to other research conducted in vitro and in rodent models (Brown et al., 2000; Wong et al., 2001b; Deloncle et al., 2006) on which this hypothesis was built. Thus, the lack of detectable changes in PrPc fails to support the hypothesis that a decrease in brain Cu concentration with a concomitant increase in Mn concentration results in an Mn for Cu substitution on PrPc that alters the biochemical properties of the prion proteins. This is in agreement with other studies we have conducted (Legleiter et al., 2007a,b) as well as that of other researchers who have questioned the importance of PrPc in Cu metabolism and the antioxidant defense system (Waggoner et al., 2000; Hutter et al., 2003; Jones et al., 2005).

It is possible that the Mn concentrations required to elicit biochemical changes in PrPc in vitro may not be physiologically possible in vivo, particularly in the bovine via oral consumption of excess Mn. Alternatively, as discussed previously (Legleiter et al., 2007b), inhalation of Mn may produce significantly greater brain Mn concentrations. Further, the PrPc-bound Cu in vivo may not be readily replaced by Mn ions, as has been reported to occur in vitro (Lehmann; 2002; Deloncle et al., 2006). Thus, the hypothesized relationship among Cu, Mn, and prions may need to be reevaluated in the context of whole-animal biology.

The hypothesis tested in this study was based on compelling observational (Purdey, 2000; Thackray et al., 2002) and experimental evidence (Brown et al., 2000; Wong et al., 2001a; Deloncle et al., 2006) implicating an imbalance in brain Cu and Mn to biochemical changes in prion proteins that are relevant to TSE, particularly sporadic TSE. To our knowledge, this is the first study that has evaluated the relevancy of previous findings regarding Cu, Mn, and prions, by exposing young calves to Cu deficiency or Cu deficiency plus high dietary Mn. We have recently conducted similar studies using mature cows; however, using young calves allowed the hypothesis to be tested in animals that were still developing and that possibly were more susceptible to perturbations in brain Cu and Mn and changes in prion biology, particularly because Cu is critical for brain development (Prohaska and Bailey, 1993; Prohaska, 2000).

In conclusion, an imbalance in dietary Cu and Mn, particularly increased concentrations of Mn in Cu-deficient calves, can dramatically alter obex Cu concentrations and Mn:Cu ratios, but does not change the biochemical properties of prion proteins. This study, in conjunction with others recently conducted (Legleiter et al., 2007a,b), collectively questions the theory that dietary Cu and Mn are involved in the pathogenesis of TSE by altering PrPc biology.

LITERATURE CITED


