ABSTRACT

LEGLEITER, LEON R. The relationship between copper, manganese, and bovine brain prion proteins: Implications for trace mineral nutrition and bovine spongiform encephalopathy. (Under the direction of Dr. Jerry W. Spears.)

The purpose of this research was to evaluate the relationship between dietary copper and manganese and bovine brain prion proteins. Aberrant prion proteins are the causative infective agent in bovine spongiform encephalopathy and other transmissible spongiform encephalopathies. While the cellular prion protein is purported to bind copper ions, a substitution of copper with manganese on the prion protein causes alterations in the biochemical properties of the prion that are similar to the infective prion. An imbalance in brain copper and manganese that allows for prion bound copper to be replaced by manganese has been implicated in the spontaneous conversion of cellular prions to aberrant prions and the subsequent sporadic forms of transmissible spongiform encephalopathies. Until now these findings have not been tested in the bovine. Thus, the studies reported here were designed to test the hypothesis that a copper deficiency, alone or coupled with high dietary manganese, would result in decreased brain copper and increased brain manganese and subsequently alter the biochemical properties of prion proteins. Considerations were given to animal age and length of exposure as well as imbalances in dietary copper and manganese. We report that brain copper is decreased in copper deficient cattle and brain manganese is slightly increased when exposed to high dietary manganese. The perturbations in brain copper and manganese did not alter prion biochemical properties. Most notably there was no apparent manganese for copper substitution on prion proteins that resulted in increased protease resistance or decreased superoxide dismutase-like activity. Also, the prion associated copper and superoxide dismutase-like activities were

significantly lower than values reported from in vitro research. Taken together, these results do not support the hypothesis that perturbations in brain copper and manganese induce biochemical changes to prion proteins. Additionally, these data suggest the relationship between brain prions and copper need to be revaluated in the context of whole animal biology.

THE RELATIONSHIP BETWEEN COPPER, MANGANESE, AND BOVINE BRAIN PRION PROTEINS: IMPLICATIONS FOR TRACE MINERAL NUTRITION AND BOVINE SPONGIFORM ENCEPHALOPATHY

by

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DEDICATION

This work is dedicated to my wife Mendy and daughter Riley. Completing a Ph.D. has been a rewarding challenge; however, nothing gives me more pride and personal satisfaction than my family. Mendy has enthusiastically supported and encouraged me throughout graduate school and has no doubt significantly contributed to any success I have had and will have in the future. The birth of our daughter Riley has unimaginably and profoundly changed my perspective on life; so much so that no career oriented success will ever match the pride and joy of that associated with raising our daughter. Thank you both for your unwavering support and love and the joy that you bring to my life.

BIOGRAPHY

Leon Ray Legleiter is the son of Belvin and Ardis Legleiter and a native of Nevada, Missouri. Leon, along with his brothers and sister, grew up on a diversified crop and livestock family farm in southwest Missouri. After graduating from Nevada High School in 1997 Leon attended the University of Missouri and earned a Bachelor of Science in Animal Science in 2001. On June 30, 2001, Leon married Mendy Caldwell, also from Nevada, MO. Leon then remained at the University of Missouri to further his education by pursuing a Master of Science in Animal Science. Leon and Mendy moved to Raleigh, North Carolina in August of 2003 so that Leon could pursue a Doctorate of Philosophy in Nutrition in the Department of Animal Science and Interdepartmental Nutrition Program at North Carolina State University. In his spare time Leon enjoys golf, hunting, traveling, and spending time with family and friends. Leon and Mendy are most recently the proud parents of daughter Riley Rae Legleiter, born June 2, 2006, in Cary, NC.

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Simply stated, the farm crew at the Butner Beef Cattle Research Center is second to none. I am sincerely grateful for all the hard work put forth by the staff to ensure reliable, timely, and high quality research studies were conducted. I have accomplished significantly more work than I thought possible in my short tenure at NCSU, largely because of the significant effort put forth by

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I would also like to thank my committee members, Dr. Liu, Dr. Odle, and Dr. Hanson for challenging me as well as supporting me throughout my work.

Lastly, I feel fortunate to have come from a strong and supportive family. To my parents and siblings, thanks for all of your support, encouragement and love. I am also truly grateful for the Caldwell family's support and love. To Mendy and Riley, thanks for all of your love and support. I am very fortunate to have you both in my life.

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LIST OF ABBREVIATIONS

AA	Atomic Absorption
BSE	Bovine spongiform encephalopathy
BW	Body weight
CD	Circular dichroism
CJD	Creutzfeldt-Jakob disease
Cu	Copper
CWD	Chronic wasting disease
DAO	Diamine oxidase
DM	Dry matter
ELISA	Enzyme-linked immunsorbent assay
IP	Immunoprecipitation
MBM	Meat and bone meal
Mn	Manganese
Mo	Molybdenum
MOPS	3-(*N*-morpholino) propane sulfonic acid
MW	Molecular weight
PAGE	Polyacrylamide gel electrophoresis
PK	Proteinase K
PrP^{c}	Cellular prion protein
PrP^{sc}	Scrapie (infective isoform) prion protein
PVDF	Polyvinylidene difluoride
$rPrP^{c}$	Recombinant cellular prion protein
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
TSE	Transmissible spongiform encephalopathy
WB	Western blot
Zn	Zinc

CHAPTER 1

Literature Review

Prion Proteins: "A historical perspective"

The first clue regarding the infectious agent involved in transmissible spongiform encephalopathies (TSE), a family of fatal neurodegenerative diseases of humans and animals, was accidentally uncovered when thousands of sheep and goats in Scotland were vaccinated against louping ill virus with a formalin-treated suspension of ovine brain and spleen. Two years later 1,500 of the vaccinated animals developed scrapie, indicating that the animals had inadvertently been inoculated with the scrapie infective agent (Gordon, 1946). This important discovery revealed the infective agent was resistant to inactivation by formalin, which rejected the hypothesis that the disease was caused by a 'slow virus' (Hadlow, 1959), as most viruses are readily inactivated by formalin treatment. Further, Alper et al. (1967) demonstrated that the infectious agent was resistant to ultraviolet and ionizing radiation, treatments that normally destroy nucleic acids. Additionally, numerous researchers have unsuccessfully attempted to find a virus responsible for the TSE (Soto, 2006).

Many other theories arose to describe the scrapie agent (Prusiner, 2004), but the protein only hypothesis, first enunciated by Griffith (1967) and later developed by Prusiner (1982), has gained the most widespread support. Research is continuing to support the Prusiner hypothesis (Prusiner, 1982), which implicates abnormal prion proteins as the infectious agents in TSE.

> *"Because the novel properties of the scrapie agent distinguish it from viruses, plasmids, and viroids, a new term "prion" is proposed to denote a small proteinaceous infectious particle which is resistant to inactivation by most procedures that modify nucleic acids" S. B. Prusiner, 1982*

Prions, proteinaceous infectious particles, are unprecedented infectious particles that contain no genetic material and cause neurodegenerative diseases through an entirely novel mechanism (Prusiner, 1982). The prion hypothesis defies several well established dogmas of basic biology with regard to infectious agents and protein folding (Soto, 2006). The study of prions has garnered the attention of the global scientific community and has been so intensely investigated over the past 20 years that it has become its own field of research. Nevertheless, even as compelling evidence supporting the prion hypothesis continues to mount, it is still met with some skepticism.

Prion Protein Biological and Functional Properties: "The good prions"

While it is known that aberrant prions are involved in neurodegenerative diseases, the cellular isoforms biological role(s) has yet to be elucidated. The cellular prion protein (PrP^c; Figure 1) is comprised of 253 amino acids and is encoded by the *PRNP* gene (Cohen, 1999). Prions are glycolipid-anchored membrane-surface proteins expressed by most tissues, especially neuronal cells (Prusiner, 1991; McMahon et al., 2001). They contain up to five-octapeptide repeats,

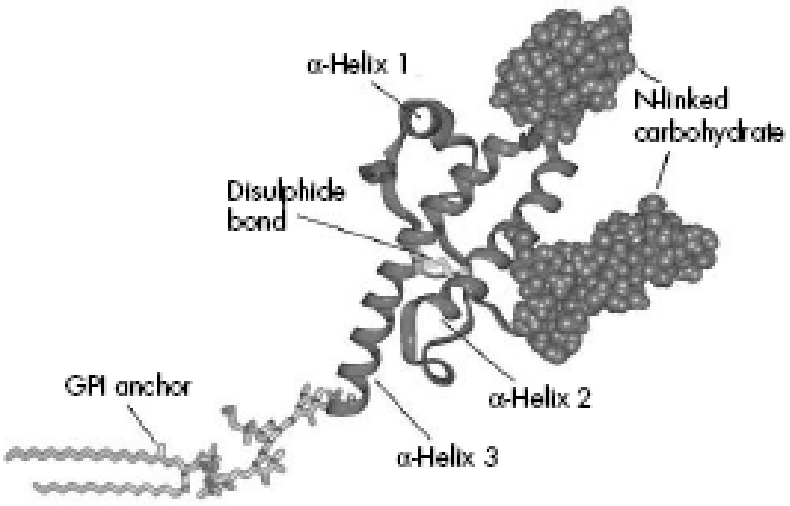

Figure 1. The human cellular prion protein (PrP^c; Collinge, 2005).

depending on the species, near the amino terminus of the protein and two glycosylation sites allowing for three glycoforms (di-, mono-, and unglycosylated; Prusiner, 1998). The octapeptide repeats (PHGGGWGQ) have the capacity to bind metal ions, particularly Cu, with an affinity in the micromolar range (Hornshaw et al., 1995a; Hornshaw et al.,1995b; Jackson et al., 2001). PrP^c is predominantly α-helical (40%) in nature and contains little β-sheeting (Pan et al., 1993).

The fact that the PrP protein is highly conserved among species would suggest it has an important function(s) (Martins et al., 2002). However, it is not clear what the functions might be since prion knockout mice appear to develop normally in some cases (Bueler et al., 1992) while exhibiting ataxia (Sakaguchi et al., 1996) and altered sleeping patterns (Tobler et al., 1996) in others. Nevertheless, research suggests PrP^c may be involved in copper uptake, protection against oxidative stress, and cell differentiation, signaling and survival (Martins et al., 2002).

Prion Proteins as Infectious Agents: "The bad prions"

The infective agent in TSE was found to be a 27-30 kD protease resistant fragment of the prion protein, which became known as PrP^{27-30}. This 27-30 kD peptide is derived from the 33-35 kD scrapie isoform of PrP,

designated PrP^{sc} (Figure 2; Prusiner, 1982; Oesch et al., 1985). It is now widely accepted that PrP^{sc} is a post-translationally modified version of PrP^{c} (Stahl and Prusiner, 1991). However, it has long been thought that the amino

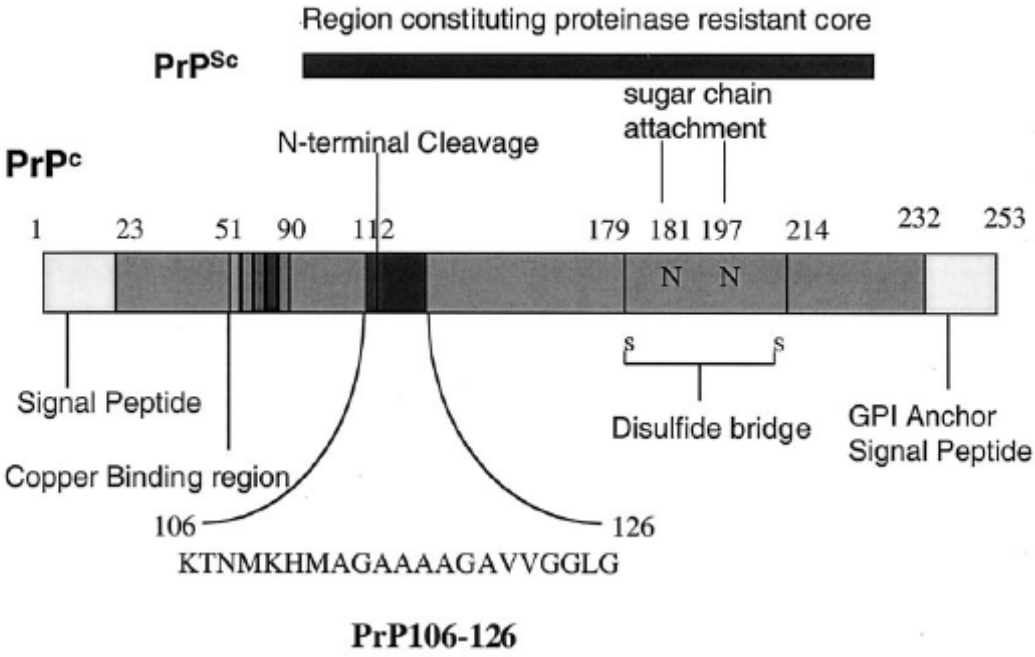

Figure 2. The primary structure of the prion protein (PrP^{c}) with the infective isoform (PrP^{sc}) region and neurotoxic peptide (PrP106-126) indicated (Brown, 2001).

acid sequence of a protein dictates its secondary structure and subsequent biologically active conformation. Therefore, the possibility that PrP^{c} and PrP^{sc} were two conformations of the same protein with the same amino acid sequence brought forth serious skepticism (Prusiner, 1998). Yet, studies using fourier-transform infrared spectrophotometry and circular dichroism (CD)

spectrophotometry showed that PrP^{c} contained about 40% α-helix and little β-sheet, while PrP^{sc}, with the exact same amino acid sequence, was composed of 30% α-helix and 45% β-sheet (Pan et al., 1993).

However, the cause and mechanisms by which PrP^{c} misfolds to the infective isoform, PrP^{sc}, is not understood. Two proposed models of replication include nucleation-polymerization and a template-assisted process (Prusiner, 2004), both of which are based on the concept that PrP^{sc} is autocatalytic. Even in the absence of cells, PrP^{sc} causes the conversion of PrP^{c} to PrP^{sc} (Raymond et al., 2000). Most importantly, PrP^{sc} incurs partial protease resistance due to the altered secondary structure. This partial resistance to protease degradation results in the formation of insoluble PrP^{sc} molecules that may polymerize into amyloid plaques (McKinley et al., 1991). The accumulation of abnormally folded PrP^{sc} in nervous tissue has been linked to TSE and at present is the only established disease marker (Wadsworth et al., 2001; DeArmond et al., 1987).

The neurotoxic mechanism(s) of PrP^{sc} has yet to be clearly elucidated; however, the pathogenesis of TSE could be the result of the loss of normal PrP^{c} function and/or direct neurotoxic effects of PrP^{sc} (Hetz et al., 2003). Evidence suggests that PrP^{c} has neuroprotective functions; therefore the loss of this protective function when converted to PrP^{sc} could impair cellular responses to oxidative stress (Kang et al., 2004). Yet, prion knockout mice did not develop prion disease either alone or in the presence of an infectious dose of PrP^{sc} (Bueler et al., 1993; Prusiner et al., 1993), thus rejecting the loss-of-function

hypothesis. However, others found prion knockout mice to develop ataxia, altered sleep-wake cycles, and altered synaptic behavior (Sakaguchi et al., 1996; Collinge et al., 1994), indicating a loss-of-function may at least be partially involved.

A prion protein peptide fragment, from the highly conserved residues 106-126 ($PrP^{106-126}$; Figure 2), was found to be highly neurotoxic (Forloni et al., 1993). This is similar to the neurotoxicity of amyloid-β in Alzheimer's disease (Brown, 2005). Although the exact mechanism(s) of $PrP^{106-126}$ neurotoxicity is not known, it may exert neurotoxic effects by inducing microglia activation (Giese et al., 1998), triggering apoptosis cascades (Lucassen et al., 1995), and increasing oxidative stress (Brown, 2005). Hetz et al. (2003) concluded that TSE may arise from the combination of the neurotoxic effects of PrP^{sc} and the loss of the neuroprotective activity of PrP^{c}.

Although the mechanism(s) by which they exert their effects is not clear, the accumulation of neurotoxic PrP^{sc} results in extensive neurodegeneration that ultimately proves fatal. The typical neuropathological alterations in TSE include vacuolization of gray matter, significant neuronal loss, synaptic dysfunction, and a variable degree of PrP^{sc} accumulation that may form plaques (Wells et al., 1993; MacDonald et al., 1996). The most notable neuropathological change is extensive vacuolization which gives the brain a spongy appearance, thus the name spongiform encephalopathy (Wells et al., 1993).

Transmissible Spongiform Encephalopathies

Transmissible spongiform encephalopathies, or prion diseases, are a group of neurodegenerative disorders affecting the central nervous system in humans and animals. The most commonly known TSE are Creutzfeldt-Jakob disease (CJD) which occurs in humans, scrapie which occurs in sheep, chronic wasting disease (CWD) occurring in deer and elk, and bovine spongiform encephalopathy (BSE; Collinge, 2001). TSE have also been described in mink, felines, and exotic ungulates (kudu, nyala, oryx; Prusiner, 2004). All of these progressive degenerative diseases ultimately prove to be fatal and are characterized by long incubation periods, failure to elicit any recognizable immune response, and an aetiology that involves remarkably stable and persistent infectious agents (Haywood and Brown, 2003).

Research is continuing to support the Prusiner hypothesis (Prusiner, 1982), that in TSE the infective agents are aberrant prion proteins. The key event in the pathogenesis of TSE, or prion diseases, is the conformational conversion of PrP^{c} to PrP^{sc}, which accumulates in neural tissue due to its protease resistance (Prusiner, 1998). Prion diseases manifest as genetic, infectious, and sporadic disorders, all of which result from the misfolding of PrP^{c} to the disease causing isoform (Prusiner, 2004).

Human Transmissible Spongiform Encephalopathies. Human prion diseases include CJD, Gerstmann-Straussler-Scheinker disease, fatal familial insomnia, and kuru (Collinge, 2001). The kuru epidemic peaked in the late 1950's in Papua New Guinea, where the Fore people were likely recycling infective prion proteins during cannibalistic rituals. It is thought that the disease originated as a case of sporadic CJD, but then continued to spread via oral consumption of human tissue during cannibalistic rituals (Alpers, 1987). At its peak, kuru caused over half of the deaths, mostly women and children, in the isolated population of Fore people (Alpers and Gajdusek, 1965). The incidence of Kuru has nearly ceased since the cessation of cannibalism in the 1950's; however, occasional cases still occur in individuals exposed to some of the last cannibalistic feasts (Collinge, 2001).

Creutzfeldt-Jakob disease, first described in the early 1920's by H. G. Creutzfeldt and A. Jakob, is a rare fatal neurological disease in humans with an incidence of one case per one million deaths annually (Prusiner, 2004). Thus, based on a life expectancy of 70 years, one in 15,000 people will die of CJD (Carrell, 2004). There are three main forms of the disease: 1) sporadic cases with no known environmental source, 2) familial cases, and 3) cases transmitted from a known or presumed environmental source, such as iatrogenic cases (Brown et al., 1992). Classic or sporadic CJD usually occurs in the 45 to 75 year age group. The clinical progression is rapid, usually

resulting in death in less than 6 months. Clinical symptoms may include fatigue, insomnia, depression, weight loss, headaches, and general mental deterioration (Collinge, 2001).

In 1995 a number of CJD cases were identified in the UK with a clinicopathologic phenotype distinct from previous cases (Will et al., 1996; Wientjens et al., 1994). Most notably, age of infection ranged from 16 to 51 years with a mean of 29 years, much younger than sporadic CJD (Hill et al., 1999). Further, psychiatric disturbances were the earliest and most predominant clinical symptoms, usually resulting in an initial referral to a psychiatrist (Collinge, 2001). It is also compelling that the atypical cases of CJD were discovered only a few years after the BSE epidemic in the UK (Will et al., 1996). Using transgenic mice, Bruce et al. (1997) reported that the new variant of CJD (vCJD) was caused by the same strain of prion as BSE, and is distinctly different, based on strain typing, from sporadic CJD. At the same time Hill et al. (1997) showed that mice expressing human PrP were indeed susceptible to vCJD PrP^{sc}. More recently, transgenic mice expressing bovine PrP were highly susceptible to vCJD and had incubation times and neuropathology indistinguishable from the same mice infected with BSE PrP^{sc} (Scott et al, 1999). Although the link between BSE and vCJD may never be proven unequivocally, these findings and numerous others like them indicate the new variant of CJD is likely the result of exposure to BSE in cattle (Prusiner, 2004). As of July, 2006, there have been 161 reported cases of vCJD in the UK

(Andrews, 2006). Worldwide there have been 194 cases of vCJD reported, as of August, 2006 (EUROCJD, 2006).

A *PRNP* gene polymorphism affects the infectivity and clinical consequences of exposure to BSE PrP^{sc} (Wadsworth et al., 2004). Valine (V) at position 129 provides protection against vCJD, while individuals that are homozygous for methionine (M) are susceptible. In fact, all vCJD patients in the UK carry the 129MM genotype (Carrell, 2004). In transgenic mice expressing human PrP^{c}, those with the genotype 129MM consistently developed vCJD when exposed to BSE, however, 129VV genotypes were mostly resistant (Wadsworth et al., 2004). Interestingly, some of the transgenic mice with *PRNP* genotype 129VV, although never showing clinical signs of vCJD, developed atypical diffuse depositions of PrP^{sc} and were able to transmit the disease to 129MM mice. This has raised concerns with prion biologists that the humans with the genotype 129VV can be dormant carriers of vCJD (Carrell, 2004).

Creutzfeldt-Jakob disease can also be transmitted iatrogenically (accidental inoculation), via PrP^{sc} contaminated neurosurgical instruments, dura mater and corneal grafting, and from injections of cadaver-derived growth hormone (Collinge, 2001). Infective prions are extremely resilient pathogens that are resistant to most cleaning and sterilization techniques (Rutala and Weber, 2001). Additionally, PrP^{sc} have the ability to bind to metal and plastic surfaces without losing infectivity (Weissmann et al., 2002). Further, Lipscomb

et al. (2006) found that two biochemical tests commonly used to confirm the efficacy of hospital surgical disinfection procedures were inadequate in detecting prion proteins. They demonstrated that up to 6.5 ug of proteinaceous brain material could remain on surgical instruments deemed clean.

The most recent concern is that iatrogenic CJD could occur via blood transfusions since it has been established that BSE can be transmitted by blood transfusion in sheep (Hunter et al., 2002). Particularly concerning is the possibility that people incubating vCJD with no clinical symptoms, especially in the UK, are donating blood and blood products (Prusiner, 2004). The first case of iatrogenic CJD, transmitted via human growth hormone treatments, was reported in 1985. As of 2006, there have been 405 confirmed cases of iatrogenic CJD (Brown et al., 2006), two times the vCJD cases caused by BSE.

Scrapie. Scrapie is the prototypical TSE and has been diagnosed in sheep and goat populations worldwide for over 200 years (Collinge, 2001). Australia and New Zealand are the only scrapie-free countries in the world (Prusiner, 2004). The common clinical symptoms of scrapie include nervousness, ataxia, weight loss, and the pruritus-induced rubbing and scratching resulting in wool loss. Interestingly, scrapie often occurs in localized epidemics that are self sustaining due to apparent horizontal transmission and environmental persistence of the infective agent (Redman et al., 2002). In fact, after persistent scrapie outbreaks Iceland tried to eradicate scrapie by re-

stocking with scrapie-free sheep, but the animals contracted the disease anyway, even on pastures that had been sheep-free for three years (Sigurdarson, 1991). In addition to horizontal transmission, vertical or maternal transmission may also be possible (Dickinson et al., 1974).

Similar to CJD, prion gene polymorphisms partially determine susceptibility and resistance to scrapie (Goldmann et al., 1991). For example, Suffolk sheep homozygous for arginine (R) at codon 171 are resistant and those heterozygous for glutamine (Q) and R at position 171 are partially resistant to scrapie. Alternatively, homozygous QQ 171 sheep are most susceptible (Westway et al., 1994). Extensive research has focused on the PrP genotype at codons 136 (A/V), 154 (R/H), and 171 (Q/R), with numerous researchers reporting complete scrapie susceptibility in animals with the VRQ allele (Prusiner, 2004). Interestingly, some breeds, most notably the Scottish blackface, seem quite resistant to scrapie, while others (Suffolk) are more susceptible (Hunter et al., 1997).

Chronic Wasting Disease. Chronic wasting disease, first documented in Colorado in 1967 by Williams and Young (1980), is a TSE affecting cervids (white-tailed deer, mule deer, and elk) in 12 states in the US (Novakofski et al., 2005). Similar to scrapie, CWD appears to be contagious via horizontal transmission (Miller et al., 2000; Gross and Miller, 2001) as epidemics in captivity and in the wild are self-sustaining (Miller and Williams, 2004). In

addition to the progressive weight loss and eventual emaciation for which the disease is named, CWD results in behavioral changes, ataxia, polydipsia, polyuria, rough hair coat, and drooping head and ears (Spraker et al., 1997; Prusiner, 2004). Pathological brain characteristics of CWD infected animals include: spongiform lesions at the obex, PrP^{sc} plaques, and significant neuronal degeneration (Williams and Young, 1993; Spraker et al., 1997). Interestingly, CWD was first thought to be a severe Cu deficiency as the brain pathology of CWD is quite similar to the spongiform encephalopathy induced by Cu deficiency (Brown, 2001). While the origin of CWD is unknown, some propose that it may have originated from scrapie (Hamir et al., 2004) while others suggest that it is a sporadically occurring TSE (Miller and Williams, 2004).

Bovine Spongiform Encephalopathy. In 1986 the first cases of BSE were discovered at the Veterinary Laboratories Agency, Weybridge, UK. The neurological disease bore a striking resemblance to that of scrapie in sheep (Wells et al., 1987). From 1986 through 2004 over 180,000 confirmed cases of BSE were identified in the UK (O.I.E., 2005). However, many more cattle may have been infected. The mean incubation period for BSE is 5 years, but most cattle are slaughtered before 5 years of age, thus many cattle were likely infected prior to developing clinical symptoms. Based on this demographic pattern, Anderson et al. (1996) estimated that nearly one million cattle were infected with BSE during the UK epidemic.

It is relatively clear that the BSE outbreak in the UK was a common-source epidemic caused by feeding ruminant by-products, primarily meat and bone meal (MBM), infected with PrP^{sc} (Nathanson et al., 1997). The outbreak of BSE in the UK reached epidemic proportions from 1986 to 1991 due to the recycling of contaminated material in the ruminant food chain (Anderson et al., 1996). However, the origin of the infective BSE agent is unknown. It may have started as a sporadic form of BSE, as an adapted strain of scrapie, or as a new variant of scrapie (Novakofski et al., 2005). Further, changes in rendering industry practices may have contributed to the spread of the BSE infective prions. In the early 1980's the common practice of solvent extracting fat from MBM ceased due to rising energy costs and diminishing profit margins between tallow and MBM (Nathanson et al., 1997). Although PrP^{sc} are notoriously resistant to degradation, lipid solvents can inactivate them (Ernst and Race, 1993). With the removal of the solvent extraction step in the processing of MBM, the hydrocarbon organic solvents were no longer present to inactivate the infective prions. This change in the production of MBM likely accelerated the BSE epidemic by recycling PrP^{sc} prior to the recognition of the disease (Prusiner, 2004). The epidemic peaked in 1992 and has since diminished, largely due to the 1989 ban of specified bovine offal in ruminant feeds.

Common clinical symptoms of BSE include fearful or aggressive behavior, ataxia, hypersensitivity, and weight loss (Wilesmith et al., 1988; Cockcroft, 2004). Extensive vacuolization of the central nervous system with

subsequent neuronal degeneration are the most common pathologic changes in BSE (Wells and Wilesmith, 1995). The incubation period may be dose dependent, but likely ranges between 3 and 5 years (Anderson et al., 1996). Once clinical symptoms manifest, the disease rapidly progresses (1-6 months) and animals become impossible to handle, thus necessitating their euthanasia (Wilesmith et al., 1988).

While no epidemics of such magnitude have occurred outside of the UK, BSE has currently been discovered in 23 other countries (O.I.E., 2005). In 2003 the first North American case of BSE was found in Canada, soon followed by the first confirmed case of BSE in the US. To date, three cases of BSE have been confirmed in the US (Hall et al., 2006).

Recently, atypical cases of BSE have been documented worldwide. Among others, France (Biacabe et al., 2004), Italy (Casalone et al., 2004) and Germany (Buschmann et al., 2006) have reported atypical BSE. Also, the two most recent cases of BSE in the US (Texas and Alabama cases) were atypical (Hall et al., 2006). While the first US BSE case had pathological characteristics similar to UK BSE cases, the two most recent US cases were quite different (Hall et al., 2006). These atypical cases had no definitive histological lesions, weak immunohistochemical staining, and unusual Western blot banding patterns. Similar to sporadic CJD in humans, there also appears to be no specific infection event (Brown, 2005). These observations suggest that BSE in

cattle exists in multiple strains and some may occur sporadically (Novakofski et al., 2005).

While BSE had been impacting the US beef industry for two decades after the disease was first discovered in the UK (Coffey et al., 2005), the first confirmed cases in Canada and the US significantly affected the economic landscape of the American beef industries (Mathews et al., 2006). The US export market suffered the largest economic impact, with Japan, Korea, and Mexico no longer accepting US beef (Mathews et al., 2006). In 2004 alone, the US beef industry lost an estimated $3.2 to $4.7 billion in exports (Coffey et al., 2005). Fortunately for the US beef industry, the export markets only account for approximately 10% of total sales (Mathews et al., 2006).

The by-product and rendering industries were also significantly affected by the discovery of BSE in the US. Increased regulations and restrictions designed as BSE safeguards increased costs of production and loss of products and/or uses (Mathews et al., 2006). The 1997 feed ban imposed by the FDA was estimated to have cost the US $53 million (USHHS) while the newest regulations after the discovery of the first case of BSE (December, 2003) cost the beef industry approximately $200 million in 2004 (Coffey et al., 2005).

However, the severity of the economic impact of BSE on US markets after the disease was discovered in the US was mitigated by four market factors: 1) the US cattle inventory was low; 2) US demand for high quality beef

was strong; 3) the discovery of BSE and subsequent economic impacts were less severe and shorter in duration than in other countries; and 4) consumption of beef by US consumers did not drastically change (Mathews et al., 2006). While economic consequences were indeed great, it is difficult to quantify the impact of BSE in monetary terms alone, particularly if BSE is indeed linked to vCJD.

Implicating Perturbations in Cu and Mn in Transmissible Spongiform Encephalopathies

Copper and prion diseases were first linked when Pattison and Jebbett (1971a; 1971b) noticed striking similarities in the histopathology of scrapie in mice and Cuprizone induced spongiform encephalopathy. Cuprizone, a Cu chelator, had depleted brain Cu and caused similar spongiform lesions and gliosis to that of scrapie infected mice. Sulkowski (1992) first hypothesized that the conformational changes that occur in the spontaneous conversion of PrP^{c} to PrP^{sc} are induced by the coordination of transition metal ions to the octa-peptide repeats. Sulkowski's hypothesis was based on the positive correlation between the multiplicity of octa-peptide repeats and susceptibility to prion disease (Goldfarb et al., 1991) and the presence of histidine residues in the octa-peptide repeats that likely serve as ligands for the binding of metal ions.

Recently, an interest in the role of metal ions, particularly Cu and Mn, in TSE has reemerged (Lehmann, 2002). Several researchers have demonstrated that a relationship exists between Cu and the biology of PrP. It has been established that PrP^c cooperatively binds Cu ions (up to five atoms) with an affinity in the micromolar range (Brown et al., 1997a; Brown, 1999; Viles et al., 1999; Kramer et al., 2001). This cooperative binding of multiple Cu ions

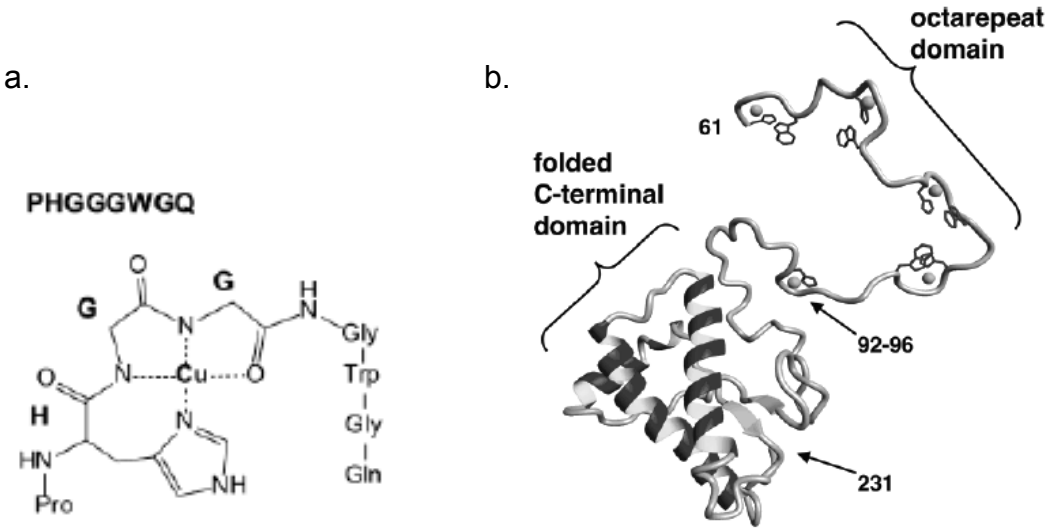

Figure 3. Prion protein binds Cu ions. a. Bond-line model of the proposed Cu coordination for each of the octapeptide repeats (PHGGGWGQ) of PrP^c (Burns et al., 2003). b. Three-dimensional rendering of PrP^c with bound Cu ions in the octapeptide repeat domain (Burns et al., 2003).

is a function of imidazole side chains of histidine residues in the several highly conserved octapeptide repeats (PHGGGWGQ, human consensus sequence) near the amino terminus of the prion protein (Figure 3; Jackson et al., 2001; Burns et al., 2003).

Interestingly, synthetic peptides mimicking the octapeptide repeats had a neuroprotective effect against Cu toxicity in cerebellar neuron cultures, particularly cultures of prion-free cerebellar neurons (Brown et al., 1998). Using an antibody to block the octapeptide repeats effectively decreased the peptides ability to bind Cu and allowed for increased Cu toxicity in the cultures. This demonstrated that the octapeptide could bind Cu and may act as a neuroprotective Cu chelator.

The binding affinity of PrP^c for Cu has been controversial and has raised questions regarding the physiological relevancy of a micromolar affinity. It is also difficult to interpret the physiological relevance of data from binding studies using peptides and recombinant proteins (Brown, 2001; Lehmann, 2002). However, a micromolar affinity is within the physiological range of Cu concentrations in plasma, cerebrospinal fluid, and synapses (Lehmann, 2002). Further, based on PrP^c mediated Cu uptake into cells, the proteins affinity for Cu may even be in the nanomolar range (Brown, 1999).

Hornshaw et al. (1995a; 1995b) demonstrated that octapeptide repeats binding Cu ions resulted in modified secondary structure of the prion protein. Using CD spectrophotometry, Garnett and Viles (2003) found that PrP^c

octapeptide repeats bind Cu ions in a coordinated manner that results in a stabilized geometry. It has been proposed that this Cu induced structuring of the amino-terminal tail may be important for acquisition of function (Lehmann, 2002).

One such function of PrP^c may be to act as an antioxidant since the protein has a superoxide dismutase (SOD)-like activity (Brown et al., 1997a; Brown et al., 1999; Wong et al., 2000). Neural tissues had an altered response to oxidative stress when PrP^c was not expressed, both *in vitro* (Brown et al., 1997a) and *in vivo* (Klamt et al., 2001; Wong et al., 2001c; Brown et al., 2002). Further, the purported PrP^c SOD-like activity appears to be Cu-dependent because prion proteins had decreased SOD-like activity when refolded in the presence of decreasing amounts of Cu ions (Brown et al., 1999). In cell culture, neurons from prion knockout mice are more sensitive to both oxidative stress and copper toxicity than wild-type neurons and have decreased activity of Cu/Zn SOD (Brown et al., 1997b). These findings suggest that PrP^c may have a protective effect against metal induced toxicity and oxidative stress (Lehmann, 2002). Further, PC12 cells, resistant to Cu and superoxide-mediated toxicity, have increased PrP^c expression, which may serve to trap Cu for cellular metabolism (Cu/Zn SOD) or prevent it from causing free Cu ion-induced oxidative damage (Brown et al., 1997c). Thus, Cu appears to be integral for normal PrP^c. If Cu is unavailable or is removed from PrP^c, it may contribute to the pathogenesis of TSE.

It has also been shown that Mn can be incorporated into native PrP^c from cells cultured with Mn enriched medium (Brown et al., 2000), and may be possible *in vivo* (Brown, 2001). However, an imbalance in Cu and Mn that allows for Mn ions to replace Cu on the octapeptide repeats may impair the function of PrP^c as an antioxidant molecule (Lehmann, 2002). In cultured astrocytes, a Mn for Cu substitution on PrP^c resulted in a destabilization of the protein. This destabilization caused the protein to misfold resulting in a loss of function and the development of partial proteinase resistance (Brown et al., 2000; Brown, 2001). The authors claimed to have demonstrated the first mechanism by which proteinase-resistant native prions can be expressed in cells. If true, this finding has significant implications as protease resistance in the hallmark characteristic distinguishing PrP^c from PrP^{sc}. Using near-infrared spectrophotometry, Tsenkova et al. (2004) demonstrated that the replacement of Cu ions with Mn on PrP^c caused a destabilization of the molecule in aqueous solution that allowed for the formation of fibrils. More recently, it was demonstrated that Mn could replace Cu on bovine PrP^c in bovine brain tissue homogenates under reducing conditions and in the presence of free radicals. This Mn for Cu substitution caused the once protease sensitive prions to become protease resistant, thus causing the once BSE negative bovine brain tissue to test positive for BSE (Deloncle et al., 2006). The authors concluded that an impairment in brain metal homeostasis that allows for increased oxidative stress may be involved in TSE.

However, it has yet to be proven that Mn loaded PrP^c, although resistant to protease degradation, is infective. Rather, Lehmann et al. (2002) proposed a model suggesting metal ions act as cofactors in the generation of PrP^{sc} through the generating of an intermediate molecule. Similarly, Cohen and Prusiner (1998) hypothesized that the conversion of PrP^c to PrP^{sc} requires the formation of "seeds" or "nuclei" of protease resistant PrP. The incorporation of Mn in place of Cu on PrP^c may be a way in which these "seeds" can form (Brown, 2001).

Additionally, metal ion imbalances have been discovered in neural tissues of TSE infected humans and animals, further supporting the hypothesis that an imbalance in Cu and Mn is linked to TSE. In CJD patients, brain Cu is decreased up to 50% and Mn increased up to ten-fold (Wong et al., 2001a). In scrapie-infected mice, brain Cu concentrations were decreased by 60% and oxidative stress markers significantly increased (Wong et al., 2001b). Likewise, in another study using scrapie infected mice, brain Cu and Mn significantly changed prior to the onset of clinical symptoms (Thackray et al., 2002). Whether these imbalances are partially causative or simply a result of the prion disease is unclear.

Lastly, ecosystems that support clusters of TSE have been documented around the world (Purdey, 2000; 2001). The common thread linking these TSE supporting ecosystems is the presence of low Cu food sources and high dietary or environmental Mn. For example, sporadic CJD and scrapie occur at high

incidence rates in a region of western Slovakia (Purdey, 2000). The affected villages are directly downwind of several ferromanganese mines and manufacturing facilities that have subsequently increased environmental Mn exposure to both humans and animals. Scrapie clusters have also been documented in Northern Iceland where the incidence is as high as 90% (Purdey, 2000). Interestingly, herbage Mn concentrations are 2.5 times higher in scrapie infected areas compared to virtually scrapie-free localities not far away. Other apparent TSE supporting ecosystems implicated in CJD, scrapie and CWD have been documented (Purdey, 2000; 2001). These observational findings by Purdey further support the hypothesis that an imbalance in Cu and Mn is involved in the pathogenesis of TSE.

Overall, the research and observational findings discussed above provide compelling evidence linking Cu and Mn to the sporadic and mysterious conversion of PrP^{c} to PrP^{sc}, and subsequent neurodegenerative TSE. However, there is also substantial evidence against this hypothesis. Firstly, the encephalopathy induced by Cuprizone in mice, while similar to scrapie in many respects, was not transmittable (Pattison and Jebbett, 1973) and was likely not scrapie. More recently, the role of PrP^{c} in brain Cu metabolism was questioned by Waggoner et al. (2000). Using mice expressing 0, 1, and 10 times the normal level of PrP^{c}, they found no correlation between PrP^{c} expression and brain Cu content or cuproenzyme activities. Subcellular fractions of brain, particularly synaptosomal fractions in which PrP^{c} is concentrated, had similar

Cu concentrations across all PrP^c expression levels. Further, the enzymatic activities of SOD and cytochrome oxidase, both cuproenzymes, were not affected by PrP^c expression. The authors concluded that the role of PrP^c in copper metabolism needed to be re-evaluated.

The relationship between PrP^c and Mn has also been questioned. Using peptide fragments representing various lengths of the octapeptide repeat region of PrP^c and CD spectral analysis, Garnett and Viles (2003) demonstrated the octapeptide repeat region cooperatively binds Cu ions but not Mn. Further, Mn ions were unable to displace PrP^c-bound Cu ions, even at high levels of Mn (0.4 mM). The discrepancy between their findings and others reporting the ability of PrP^c to bind Mn is likely a function of the assay techniques used. Garnett and Villes (2003) used CD spectrophotometry whereas all other reports have been based on equilibrium dialysis.

The SOD-like activity of PrP^c has been refuted by Hutter et al. (2003) and Jones et al. (2005). Hutter et al. (2005) crossed mice lacking to *Sod1* gene, which encodes for SOD-1, with mice variably expressing the *Prnp* gene, which encodes for PrP^c, and found no correlation between prion protein expression and SOD activity. However, when mice, both lacking and expressing PrP^c, were crossed with mice expressing various levels of SOD, they found SOD activity to be exclusively correlated with *Sod1* gene expression. They concluded that PrP^c makes no discernable contribution to total SOD activity. Jones et al. (2005) found that recombinant PrP^c ($rPrP^c$) refolded in the

presence of Cu has no more SOD activity than Cu ions in water. Taken together, these studies conclude that PrP^c has no SOD-like activity *in vitro* or *in vivo*.

Copper Metabolism in the Bovine

Copper absorption in ruminants in low (1-10%), primarily due to the presence of strong antagonists that bind Cu in the ruminal environment (Underwood and Suttle, 1999). Once absorbed, Cu is stored in the liver, incorporated into ceruloplasmin or other cuproenzymes, or excreted in bile. Copper is circulated to extrahepatic tissues via ceruloplasmin and albumin (Cousins, 1985). Copper uptake by cells and transport intracellularly is mediated by a host of Cu transporters and chaperones (Bertinato and L'Abbe, 2004). Copper ions serve as important catalytic cofactors in redox chemistry because of their ability to adopt distinct redox states (Pena et al., 1999). Important cuproenzymes include Cu/Zn SOD, cytochrome oxidase, lysyl oxidase, tyrosinase, and ceruloplasmin.

Copper deficiency in the bovine, a widespread problem in many areas of North America, may result in decreased growth, anemia, weak bones, cardiac failure, depigmentation of hair, and reduced reproductive efficiency (NRC, 1996). A Cu deficiency in ruminants can occur as a primary deficiency, where

Cu intake is inadequate, or as a secondary deficiency, whereby other factors in the diet interfere with the absorption and/or metabolism of Cu (Gengelbach et al., 1994). Copper bioavailability in ruminant diets is particularly low when molybdenum and sulfur are present in moderate to high concentrations which results in the formation of highly antagonistic thiomolybdates (Davis and Mertz, 1987; Suttle, 1991).

Copper is critically important for brain development and function, and a deficiency can result in decreased brain Cu and subsequent neurological dysfunction (Prohaska, 2000). Suttle and Angus (1976) noticed an 18% decrease in brain Cu of young calves that were Cu-depleted. Lambs born to unsupplemented ewes, compared to Cu-supplemented ewes, were born with markedly reduced brain Cu concentrations (60% decreased brain Cu, Suttle and Field, 1969; 74% decreased brain Cu, Suttle et al., 1970) that resulted in an ataxic condition known as swayback. Mature ewes that received no supplemental Cu also had decreased (64%) brain Cu (Suttle and Field, 1969). However, few studies have given attention to the effects of Cu status on brain tissue Cu in adult animals, particularly the mature bovine.

Manganese Metabolism in the Bovine

Manganese is poorly absorbed by the ruminant at a rate of 0.5 to 1% (Gibbons et al., 1976; Sansom et al., 1978). Manganese absorbed by the small

intestine is transferred to the liver bound to albumin via portal circulation (Leach and Harris, 1997). Unlike Cu, Mn storage in the liver is limited and tightly regulated via effective homeostatic mechanisms (Hall and Symonds, 1981). Consumption of high dietary Mn results in increased biliary excretion (Hall and Symonds, 1981) and decreased intestinal absorption (Abrams et al., 1977). Further, manganese is removed from blood very efficiently by the liver (Gibbons et al., 1976; Legleiter et al., 2005). In some cases feeding very high levels of Mn has slightly increased liver and extrahepatic tissue Mn concentrations (Watson et al., 1973; Legleiter et al., 2005); however, the biological relevancy of these increases is unknown.

Chronic exposure to Mn can lead to toxic effects on the central nervous system resulting in a Parkinson's like disease called manganism (Dobson et al., 2004). While the respiratory and gastrointestinal tracts are the primary means of entry into the body, most Mn toxicities are associated with inhalation (Oberdoester and Cherian, 1988). Most cases of manganism occur as a result of occupational hazards, usually related to mining and manufacturing (Dobson et al., 2004). Inhaled Mn is likely to reach the central nervous system before hepatic clearance, thus explaining the increased likelihood of manganism due to inhalation versus consumption (Roels, et al., 1997; Heilig et al, 2005). In rats, administering $MnCl_2$ via oral gavage and intraperitoneal injection had no effect on cerebellum and striatum Mn concentrations, but intratracheal

instillation significantly increased Mn in both tissues (Roels et al., 1997). Little is known about Mn inhalation in the bovine.

Like Cu, Mn is an integral component of multiple enzymatic reactions responsible for fundamental biological functions. Manganese containing enzymes include pyruvate carboxylase, Mn SOD, and arginase. Many other enzymes (kinases, decarboxylases, hydrolases, and transferases) can be activated by Mn (Leach and Harris, 1997).

Conclusion

Even if the mechanism(s) behind infectious TSE is elucidated, sporadic forms of the diseases will likely continue to occur with no know infectious event (Brown, 2001; Brown, 2005; Novakofski et al., 2005). An environmental cause seems plausible, especially considering observational evidence of clusters of TSE occurring in low Cu, high Mn regions of the world (Purdey, 2000). Coupling these environmental observations with compelling data from controlled experiments supports the hypothesis that an imbalance in environmental Cu and Mn that allows for a Mn for Cu substitution of PrP^c is a causative factor in sporadic TSE (Brown, 2001). While it is relatively clear that the BSE epidemic in the UK was a function of feeding infective ruminant by-products, the original disease may have arisen sporadically (Brown et al.,

2000), as is known with sporadic CJD (Prusiner, 1991), and may be related to brain tissue metal ion imbalances (Brown et al., 2000). Further, this hypothesis regarding Cu, Mn, and prions is especially intriguing in light of recently documented atypical cases of BSE.

Most research in prion biology, including that mentioned above, has been conducted *in vitro* or *in vivo* using rodent models. Moreover, outside of post-mortem observations in infected animals, most BSE research has been conducted in the mouse. To our knowledge no research has been conducted using the bovine to examine the relationships between Cu, Mn, and bovine prion proteins *in vivo*. It is imperative that research be conducted using the bovine animal to verify the results of previous studies using *in vitro* techniques and rodent models, especially with regard to BSE. Thus, the research reported here tests the hypothesis that a Cu deficiency or Cu deficiency coupled with high dietary Mn will alter brain Cu and Mn concentrations and affect the biochemical properties of prion proteins.

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CHAPTER 2

Bovine copper deficiency results in decreased brain copper but has no apparent effect on prion proteins characteristics[1]

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Abstract

Aberrant prion proteins (PrP^{sc}) are the infective agent in transmissible spongiform encephalopathies (TSE), while cellular prion proteins (PrP^{c}) appear to function as copper (Cu) transporters, cellular messengers, and antioxidant molecules. Although the function of PrP^{c} is controversial, Cu appears essential for normal prion biology. The removal of Cu has been implicated in altering PrP^{c} biochemical characteristics. Eight mature Angus cows were used to determine the effects of Cu deficiency on brain Cu concentrations and PrP^{c} functional characteristics. Copper deficiency was induced by feeding a low-Cu diet supplemented with 5 mg Mo/kg DM and 0.3% S for 216 d. The copper-deficient cows were randomly assigned to one of two treatments: 1) Cu-deficient (-Cu; no supplemental Cu), or 2) Cu-adequate (+Cu; 10 mg supplemental Cu/kg DM from Cu sulfate). After the 159-d repletion phase the cows were slaughtered and brain and liver samples were collected. At the end of the repletion phase +Cu cows had adequate liver Cu while -Cu cows were still Cu-deficient with significantly less ($P = 0.01$) liver Cu than the supplemented cows. Copper-deficient cows (-Cu) had lower ($P = 0.007$) brain Cu concentrations than +Cu cows. Brain PrP^{c} concentrations, based on relative optical density and ELISA analysis, were similar across treatments. Based on Western blot analysis the molecular weights, glycoform distributions, and elution profiles of brain PrP^{c} were not affected by Cu status. Copper status

did not affect PrP^c proteinase degradability as all prions were completely degraded by proteinase K. Total superoxide dismutase activity of the brain tissue homogenates was not affected by brain Cu concentrations. In conclusion, Cu status affected bovine brain Cu concentrations but had no apparent effects on brain prion protein characteristics.

Keywords: Bovine, Brain, Copper, Prion protein

Introduction

Prions, proteinaceous infectious particles, are the infectious agents in transmissible spongiform encephalopathies (TSE; Prusiner, 1982). The family of TSE includes Creutzfeldt-Jakob disease (CJD), scrapie, chronic wasting disease (CWD) and bovine spongiform encephalopathy (BSE), among others (Collinge, 2005). These fatal neurodegenerative diseases manifest as genetic, infectious, or sporadic disorders, all of which result from the misfolding of the cellular prion protein (PrP^{c}) isoform to the infective and disease causing isoform (PrP^{sc}; Prusiner, 2004) that is partially resistant to proteinase K (PK) degradation (Prusiner et al., 1998).

Several researchers have suggested metal ions, in particular copper (Cu), may be involved in TSE (Brown, 2001; Lehmann, 2002). Pattison and Jebbett (1971a; 1971b) were the first to propose a link between prion disease and Cu deficiency, when they observed striking histological similarities in scrapie infected mice and cuprizone induced Cu-deficient mice. It has since been established that PrP^{c} cooperatively binds Cu ions with an affinity in the micromolar range (Brown et al., 1997a; Brown, 1999; Kramer et al., 2001). Hornshaw et al. (1995) demonstrated that octapeptide repeats binding Cu ions resulted in a stabilization of the protein that may be important for acquisition of function (Lehmann, 2002). Although the function(s) of PrP^{c} has yet to be

elucidated, several proposed functions that involve Cu have been described (Brown et al., 1999; Martins and Brentani, 2002). Most notably, PrP^c may play a key role in antioxidant defense via its Cu-dependent superoxide dismutase (SOD)-like activity (Brown et al., 1997b; Brown et al., 1999; Wong et al., 2000).

Prion proteins may also bind Mn ions (Brown et al., 2000; Brown, 2001). However, an imbalance in Cu and Mn that allows for Mn ions to replace Cu on PrP^c has led to biochemical changes in PrP^c (Brown et al., 2000; Tsenkova et al., 2004; Deloncle et al., 2006). Interestingly, large decreases in brain Cu and increases in brain Mn have been associated with TSE (Wong et al., 2001a; Wong et al., 2001b; Thackray et al., 2002).

Thus, it appears that Cu is critical for the normal function of PrP^c, and its absence may lead to biochemical changes in PrP^c that could have implications in TSE. However, the relevancy of these findings has not been tested outside of *in vitro* techniques and rodent models. To our knowledge no research has been conducted to evaluate the relationship between Cu status, brain Cu concentrations, and the biology of prion proteins in the bovine. Thus, the objective of this study was to determine the effects of Cu deficiency in the bovine on brain Cu concentrations and prion protein biochemical characteristics.

Materials and Methods

Animals and Experimental Design

To achieve two contrasting Cu states (Cu-deficient and Cu-adequate), the study consisted of a depletion phase (216 d), designed to induce Cu deficiency, followed by a repletion phase (159 d). Feeding supplemental molybdenum (Mo) and sulfur (S) allows for the formation of thiomolybdates, which tightly bind Cu and thereby greatly reduce absorption. Continual exposure to a low Cu diet coupled with high levels of Mo and S will induce Cu deficiency in cattle (Underwood and Suttle, 1999).

Eight mature Angus cows (8.2 ± 1.8 yr) were fed a low Cu (5.03 mg Cu/kg DM) diet consisting of chopped alfalfa hay and corn stalks supplemented with 5 mg Mo/kg of diet DM and 0.3% sulfur for 216 d to induce Cu deficiency. Copper status was monitored throughout the study by analyzing liver biopsies and plasma samples. Liver biopsies were performed using the true-cut technique described by Pearson and Craig (1980), as modified by Engle and Spears (2000). At the same time that liver biopsy samples were collected, blood samples were collected via jugular venipuncture into heparinized, trace mineral-free, vacutainer tubes (Becton Dickinson Co., Franklin Lakes, NJ). Blood samples were transported back to the laboratory on ice, centrifuged at 2000 x g for 15 minutes at room temperature, and plasma transferred to acid-washed polypropylene storage vials and stored at -20ºC. Cattle were

determined to be Cu-deficient when liver Cu concentrations fell below 30 mg/kg DM (Mills, 1987). Following Cu depletion, cows were stratified based on age, weight, body condition score, and liver Cu concentration and randomly assigned to one of two treatments: 1) Cu-deficient (-Cu; no supplemental Cu), 2) Cu-adequate (+Cu; 10 mg supplemental Cu/kg DM from Cu sulfate). Cattle were fed a ground alfalfa hay-based diet (7.90 mg Cu/kg DM) with the corresponding treatment provided in a corn supplement at 2% of dietary DM. All cows were individually fed to ensure individual daily intake of the assigned treatment and all cows were fed to meet NRC (1996) requirements with the exception of Cu.

At the end of the repletion phase cows were humanely euthanized via captive bolt followed by exsanguination. Cerebral tissue was harvested from each cow, wrapped in aluminum foil and immediately frozen in liquid nitrogen. A liver sample was also collected and transported back to the laboratory on ice. Samples were stored at -80ºC until analysis.

Analytical Procedures

Liver samples were analyzed for Cu concentration as described by Engle et al. (1997) and plasma Cu measured as described by Ahola et al. (2004). Brain samples for Cu analysis were prepared using a microwave digestion (Mars 5, CEM Corp., Matthews, NC) procedure described by Gengelbach et al. (1994). Approximately 0.5 g of dried tissue was added to 5 mL of trace mineral

grade nitric acid (Fisher Scientific) and allowed to digest overnight prior to microwave digestion. Copper in the ashed brain samples was determined using flame atomic absorption spectrophotometry (GFA-6500, Shimadzu Scientific Instruments, Kyoto, Japan).

Total protein was extracted from brain tissue as described by Wong et al. (2000). Approximately 1 g of chilled cerebral tissue was homogenized on ice in 9 mL of chilled extraction buffer (0.01 M PBS, 1% Nonidet P40, 10% w/v complete EDTA-free protease inhibitor cocktail tablets; Roche Diagnostics Corp.) with a Polytron homogenizer in a 50 mL polycarbonate tube. Homogenates were immediately centrifuged at 5,000 x g for 20 minutes at 4ºC and the supernatant was analyzed for total protein using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA) so that samples could be equilibrated based on protein concentration. The protein equilibrated 10% brain tissue homogenates were aliquotted into microcentrifuge tubes and stored at -80ºC until analysis.

All electrophoresis and Western Blot (WB) supplies were purchased from Invitrogen Corp. (Carlsbad, CA) unless otherwise stated. Polyacrylamide Gel Electrophoresis (PAGE) was performed using pre-cast NuPAGE Novex 10% Bis-Tris gels and the Novex X-Cell Surelock Mini-Cell electrophoresis system. Magic Mark XP Western Protein Standard molecular weight marker was used for molecular weight (MW) determination. Recombinant PrP^{c} ab753 (Abcam

Inc., Cambridge, MA) and water were used as positive and negative controls, respectively.

Proteins were separated on the gel under denaturing conditions using MOPS SDS running buffer (pH 7.3-7.7) at 200V constant for 50 minutes. After electrophoresis the gel was gently removed from the casing and the proteins were transferred to a PVDF membrane using the XCell II Blot Module and NuPAGE Transfer Buffer with 10% methanol and 0.1% antioxidant at a constant 30V for 60 minutes. The WB was visualized using the Western Breeze Chemiluminescent Kit. All washes were done at room temperature using a shaker platform. Membrane bound PrP^c were probed with 1:10,000 diluted anti-PrP^c mAb 6H4 (Prionics AG, Switzerland) for one h. After extensive rinsing the membrane was incubated with the secondary antibody, anti-mouse conjugated to Alkaline phosphatase, for 30 minutes followed by exposure to 2.5 mL of chemiluminescent substrate (CDP-Star) for 5 minutes. To capture the WB image, Kodak X-OMAT LS film (Eastman Kodak Co., Rochester, NY) was exposed to the membrane and developed using an auto-developer (Kodak X-OMAT Clinic 1 Processor, Eastman Kodak Co., Rochester, NY).

Membranes were stripped using Restore Western Blot Stripping Buffer (Pierce Biotechnology, Inc., Rockford, IL) and probed for β-actin using anti-β-actin (Sigma-Aldrich, Inc., St. Louis, MO). Membranes were visualized using chemiluminescence as previously described. Beta-actin was used as an internal loading control.

The WB images were analyzed using the Kodak 1-D Image Analysis Software (Eastman Kodak Co., Rochester, NY). Analysis included MW determination, glycoform distribution, and relative optical densitometry.

To determine the effects of Cu status on prion proteinase degradability, samples were exposed to proteinase K (PK; Bio-Rad Laboratories, Inc., Hercules, CA) as described by Thackray et al. (2002) prior to PAGE and WB. Briefly, 250 ul PK/mL 10% brain tissue homogenate was allowed to digest for 1 h at 37ºC. Proteinase degradability was determined by comparing WB elution profiles of PrP^c exposed to PK with those not exposed to PK. The inability to detect a banding pattern from samples exposed to PK indicated complete degradation by PK.

PrP^c was quantitated using a double antibody sandwich enzyme-linked immunosorbent assay kit (ELISA; Cayman Chemical Co., Ann Arbor, MI). Brain homogenates were incubated in duplicate wells on a 96 well plate pre-coated with anti-PrP^c mouse monoclonal antibody for 2 h at room temperature. A standard curve was constructed using known quantities of $rPrP^c$ ab753. Wells were rinsed with wash buffer five times followed by addition of the second anti-PrP^c antibody conjugated to acetylcholinesterase and incubated for 2 h. After thoroughly washing all wells with wash buffer, 200 ul of Ellman's Reagent was added to each well and incubated for 30 minutes in darkness at room temperature. Absorbances were read at 405 nm using a plate reader (Synergy

HT, Bio-Tek Instruments, Inc., Winooski, VT). Ellman's reagent was used as the blank and absorbances were corrected accordingly.

Total superoxide dismutase (SOD) activity of the brain tissue homogenates was measured using a SOD assay kit (Cayman Chemical Co., Ann Arbor, MI). Purified SOD was used as a positive control and to construct a standard curve for sample SOD activity quantitation. Brain tissue homogenates were mixed with 200 ul of the radical detector (tetrazolium salt) in duplicate wells. The addition of 20 ul of xanthine oxidase to each well and incubation for 20 min allowed for the formation of superoxide radicals and subsequent color formation. Absorbances were read at 450 nm using a plate reader (Synergy HT, Bio-Tek Instruments, Inc., Winooski, VT). One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radicals.

Statistical analysis

All data was analyzed as a completely randomized design using Proc Mixed in SAS (SAS Inst. Inc., Cary, NC). In the model treatment served as the fixed effect and animal was used as a random variable. Effects were considered significant at $P < 0.05$.

Results

Tissue mineral concentrations are presented in Table 1. Six of the eight cows were Cu-deficient (liver <30 mg Cu/kg DM; Mills, 1987) at the start of the repletion phase, while two (1 per treatment) were marginally Cu-deficient. At the end of the repletion phase, cows receiving treatment +Cu were Cu-adequate while the -Cu cows had significantly less (P = 0.01) liver Cu and were all Cu-deficient. Copper-deficient cows had lower (P = 0.007) brain Cu concentrations than Cu-adequate cows.

Based on WB analysis, relative optical densities of total PrP^c were not different among treatments, indicating brain Cu did not influence PrP^c concentrations (Figure 1). Likewise, using the ELISA to quantitate PrP^c, no differences were seen in prion concentrations between the two treatments with an average of 1.2 ng of PrP^c/g cerebral tissue (Figure 2). Molecular weights of the three PrP^c glycoforms were not affected by Cu status and ranged from 36.6 to 27.1 kD for the diglycosylated and unglycosylated, respectively (Table 2). Additional WB analysis showed that the glycoform distribution was unaffected by treatment (Table 2). Based on visual inspection, the banding patterns of the PrP^c after PAGE and WB were similar across treatments (Figure 3). Copper status also did not affect PrP^c proteinase degradability as all prions were completely degraded by PK and no longer visible on the WB (Figure 4). This indicates that no resistant isoforms (PrP^{sc}) were present in any of the cows and

all animals were BSE negative. Superoxide dismutase activity of the brain tissue homogenates was not affected by dietary Cu (Figure 5).

Discussion

This study demonstrated that brain Cu in mature cows is affected by their Cu status. Specifically, Cu deficiency resulted in decreased brain Cu. This is a key finding because any effects of brain Cu on prion protein biology is dependent upon the potential for changes in brain Cu concentrations to occur. Suttle and Angus (1976) noticed an 18% decrease in brain Cu of young calves that were Cu-depleted. Lambs born to unsupplemented ewes, compared to Cu-supplemented ewes, were born with markedly reduced brain Cu concentrations (60% decreased brain Cu, Suttle and Field, 1969; 74% decreased brain Cu, Suttle et al., 1970). Mature ewes that received no supplemental Cu also had decreased (64%) brain Cu (Suttle and Field, 1969). Few studies have given attention to the effects of Cu status on brain tissue Cu in adult animals, particularly mature cows. We believe this is the first report of decreased brain Cu in mature cows due to Cu deficiency. Further, the magnitude of the decrease in brain Cu, 44% less brain Cu in -Cu cows versus +Cu cows, is noteworthy.

The decreased brain Cu due to Cu deficiency had no effects on the biochemical properties of PrP^c. The demonstrated ability of PrP^c to bind Cu ions both *in vitro* (Hornshaw et al., 1995) and *in vivo* (Brown et al., 1997a), coupled with the observation that PrP^c-null mice have significantly lower brain Cu concentrations, has suggested that PrP^c plays a key role in brain Cu metabolism. However, in the present study the concentrations of PrP^c, similar to those reported by Moudjou et al. (2001) in sheep, were not affected by brain Cu content. This is in agreement with Waggoner et al. (2000), who demonstrated that mice expressing 0, 1, and 10 times the normal level of PrP^c had similar brain Cu concentrations and cuproenzyme activates, causing them to question the importance of PrP^c in brain Cu metabolism. Prions have also been demonstrated to have a Cu-dependent SOD-like activity that contributes to the total SOD activity of brain tissue (Brown et al., 1997b; Brown et al., 1999). We were unable to detect any changes in total SOD activity in the brain tissue samples, indicating that the decreased brain Cu in -Cu cows had no effect on total SOD enzyme activity or the purported SOD-like activity of PrP^c. More recent research has questioned the role of PrP^c as an antioxidant molecule, as the protein was found to have minimal, if any, SOD activity both *in vitro* (Jones et al., 2005) and *in vivo* (Hutter et al., 2003).

All other properties of PrP^c that were analyzed appeared to be unaffected by cow Cu status and subsequent brain Cu content. Significant changes in these properties would have strengthened the data supporting a

functional relationship between Cu and PrP^c. However, finding no differences in PrP^c between cows with large differences in brain Cu concentrations questions the relevancy of previously reported findings regarding PrP^c and Cu.

There is compelling data supporting a relationship between brain metal ion perturbations and TSE. Several studies have implicated a Mn for Cu replacement on PrP^c in the pathogenesis of TSE (Brown et al., 2000; Wong et al., 2001a; Thackray et al., 2002; Tsenkova et al., 2004; Deloncle et al., 2006). However, the relevancy of these findings has not been tested in the bovine. It would be interesting to examine the effects of a Cu deficiency coupled with exposure to high dietary Mn on bovine brain PrP^c. In the present study we noticed no differences in brain Mn concentrations between the Cu-adequate and Cu-deficient cows.

To our knowledge this is the first study designed to examine the relationship between bovine Cu status, brain Cu concentrations, and prion protein biology. We conclude that inducing a Cu deficiency in mature cows resulted in markedly lower brain Cu concentrations compared to Cu-adequate cows. However, the reduced brain Cu concentrations had no apparent effects on prion biochemical characteristics.

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Table 1. Effect of dietary Cu level on liver Cu and brain Cu and Mn concentrations

	Treatments[a]			
	-Cu	+Cu	SEM	*P values*
Repletion phase, start				
Liver Cu, mg/kg	33.2	38.9	10.1	0.70
Repletion phase, end				
Liver Cu, mg/kg	23.5	90.6	9.9	0.003
Brain Cu, mg/kg	5.9	13.4	1.0	0.003
Brain Mn, mg/kg	0.7	0.9	0.1	0.47

[a]Cu-deficient (-Cu); Cu-adequate (+Cu).

Table 2. Effect of dietary Cu level on prion protein glycoform molecular weights and relative distributions

	Treatments[a]			
	-Cu	+Cu	SEM	*P values*
Molecular weight, kD				
Diglycosylated	36.6	36.6	0.19	0.91
Monoglycosylated	32.3	32.1	0.15	0.58
Unglycosylated	27.1	27.2	0.17	0.91
Glycoform distribution, %				
Diglycosylated	33.8	34.6	0.67	0.42
Monoglycosylated	23.7	24.7	1.12	0.56
Unglycosylated	42.5	40.7	0.97	0.24

[a]Cu-deficient (-Cu); Cu-adequate (+Cu).

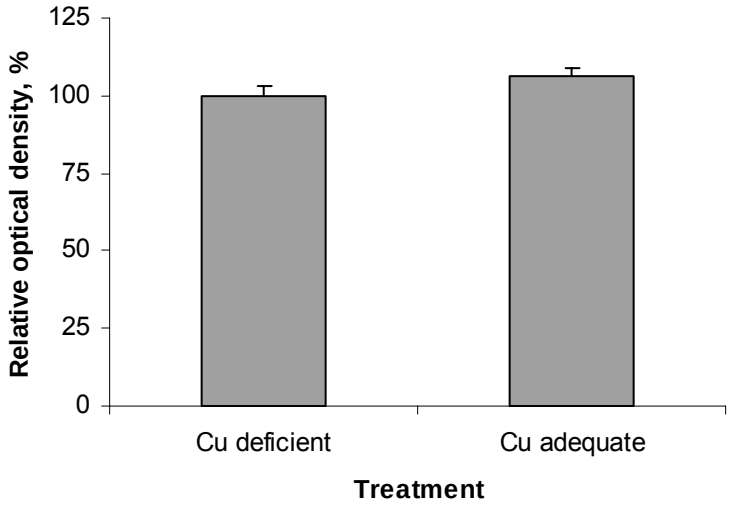

Figure 1. Effects of dietary Cu level on relative concentrations of immunoreactive prion proteins. Relative optical densities of prion protein bands were determined by densitometric analysis of Western blots. The mean relative optical density of prion protein bands from Cu-adequate cows is expressed as a percent of the mean optical density of prion protein bands from Cu-deficient cows. $P = 0.13$

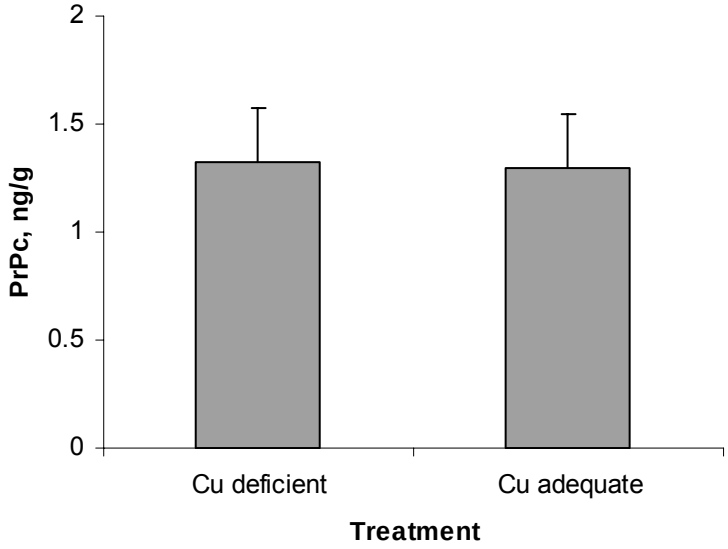

Figure 2. Effects of dietary Cu level on brain prion protein concentrations. Brain prion proteins (PrP^c) were quantitated using a double-sandwich antibody enzyme-linked immunsorbent assay. $P = 0.95$

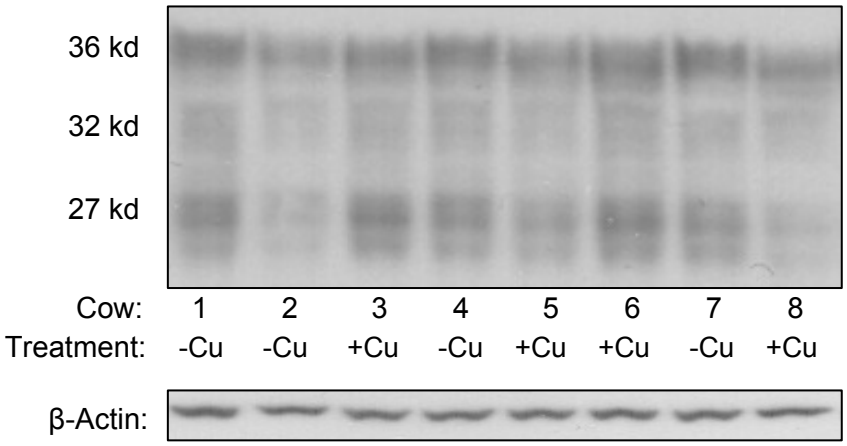

Figure 3. Effects of dietary Cu level on prion protein elution profiles. This figure is a representative Western blot of prion proteins (PrP^c) from brain tissue homogenates from all eight cows. Densitometric analysis indicated that relative optical densities of PrP^c bands were similar across treatments. Further, glycoform distributions and molecular weights were similar across treatments. Visual analysis of the blot shows similar elution profiles across treatments. β-actin was used to normalize all lanes.
Treatments: Cu-deficient (-Cu); Cu-adequate (+Cu).

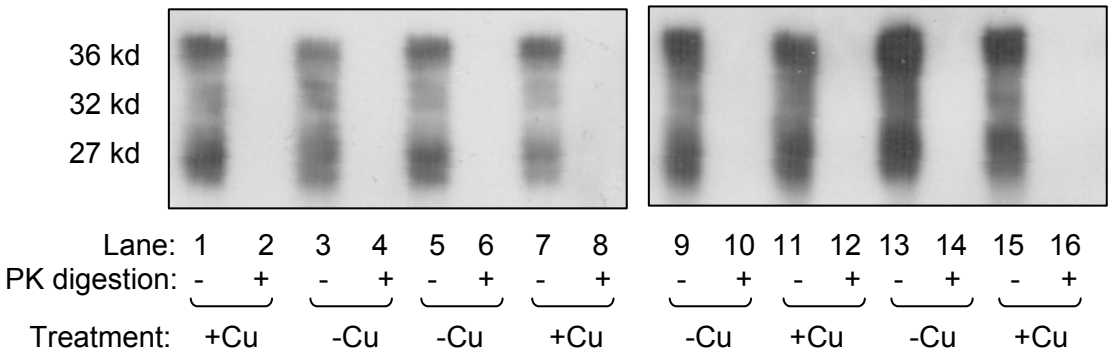

Figure 4. Effects of dietary Cu level on prion protein proteinase degradability. These two representative Western blots show immunoreactive prion proteins (PrP^c) before and after digestion by proteinase K (PK). Copper status did not affect PrP^c PK degradability as all PrP^c were completely degraded in brain tissue homogenates from all cows.
Treatments: Cu-deficient (-Cu); Cu-adequate (+Cu).

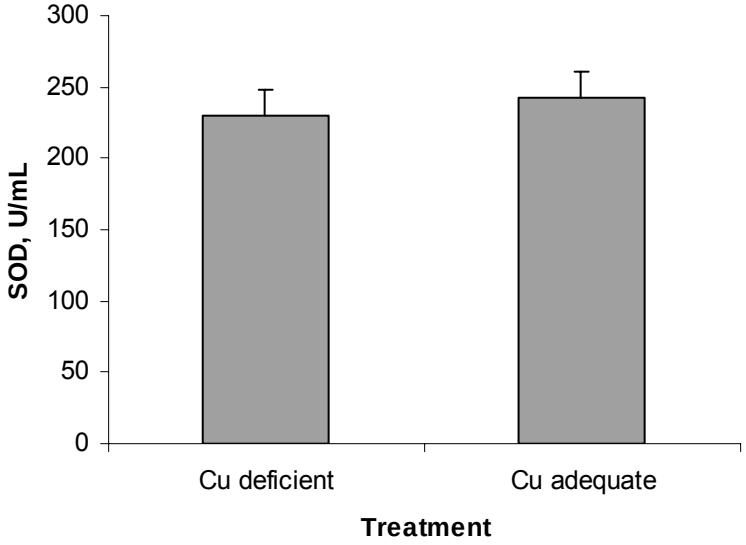

Figure 5. Superoxide dismutase (SOD) activity of brain tissue homogenates from Cu-deficient and Cu-adequate cows. The SOD activity is expressed as U/mL of protein equilibrated brain tissue homogenate. One unit (U) of SOD is defined as the amount of enzyme required to exhibit 50% dismutation of the superoxide radicals. $P = 0.67$

CHAPTER 3

Exposure to low dietary copper or low copper coupled with high dietary manganese for one year does not alter brain prion protein characteristics in the mature bovine[1,2]

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[1]Use of trade names in this publication does not imply endorsement by the North Carolina Agric. Research Serv. or criticism of similar products not mentioned.

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Abstract

Aberrant prion proteins are the causative agent in bovine spongiform encephalopathy (BSE). Cellular prion proteins (PrP^c) bind copper (Cu), which appears to be required to maintain functional characteristics of the protein. The replacement of Cu on PrP^c with manganese (Mn) has resulted in loss of function and increased protease resistance. Twelve mature cows were used to determine the effects of Cu deficiency, alone and coupled with high dietary Mn, on brain Cu and Mn concentrations, and PrP^c functional characteristics. Copper-adequate cows were randomly assigned to treatments: 1) control (adequate in Cu and Mn), 2) Cu-deficient (-Cu), and 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 molybdenum (Mo) to further induce Cu deficiency. After 360 d, copper-deficient cows (-Cu & -Cu+Mn) tended to have lower brain Cu concentrations ($P = 0.09$). Brain Mn tended ($P = 0.09$) to be higher in -Cu+Mn cattle versus -Cu cattle. Western blots revealed that PrP^c relative optical densities, proteinase K degradability, elution profiles, molecular weights, and glycoform distributions were not different among treatments. The concentration of PrP^c, as determined by ELISA, was similar across treatment groups. Brain tissue Mn superoxide dismutase activity was highest ($P = 0.04$) in cattle receiving -Cu+Mn, while immunopurified PrP^c had similar superoxide dismutase-like activities among treatments. These data

suggest that Cu deficiency coupled with excessive Mn intake in the bovine may decrease brain Cu and increase brain Mn; however, these brain metal perturbations do not significantly alter PrP^c functional characteristics.

Key Words: Bovine, Copper, Manganese, Prion

Introduction

Research is continuing to support the Prusiner hypothesis (Prusiner, 1982), that in transmissible spongiform encephalopathies (TSE), Creutzfeldt-Jakob disease (CJD), scrapie, chronic wasting disease (CWD) and bovine spongiform encephalopathy (BSE), the infective agents are abnormal prion proteins. Prions, proteinaceous infectious particles, are unprecedented infectious particles that contain no genetic material and cause neurodegenerative diseases through an entirely novel mechanism (Prusiner, 1982). Transmissible spongiform encephalopathies manifest as genetic, infectious, or sporadic disorders, all of which result from the misfolding of the cellular prion protein (PrP^{c}) isoform to the infective and disease causing isoform (PrP^{sc}; Prusiner, 2004) that is partially resistant to proteinase K (PK) degradation (Prusiner et al., 1998). The accumulation of abnormally folded PrP^{sc} in nervous tissue has been linked to TSE and at present is the only established disease marker (Wadsworth et al., 2001).

Recently, a growing interest in the role of metal ions, in particular copper (Cu), in TSE has emerged (Brown, 2001; Lehmann, 2002). Pattison and Jebbett (1971a; 1971b) were the first to notice striking histological similarities in scrapie infected mice and cuprizone induced Cu-deficient mice. It has since been established that PrP^{c} cooperatively binds Cu ions with an affinity in the micromolar range (Brown et al., 1997; Brown, 1999; Kramer et al., 2001).

Hornshaw et al. (1995) demonstrated that octapeptide repeats binding Cu ions resulted in a stabilization of the protein that may be important for acquisition of function (Lehmann, 2002). Although the function(s) of PrP^c has yet to be elucidated several proposed functions that involve Cu have been described (Brown et al., 1999; Martins and Brentani, 2002). Most notably, PrP^c may play a key role in oxidative stress homeostasis via its Cu-dependent superoxide dismutase (SOD)-like activity (Brown et al., 1997; Brown et al., 1999; Wong et al., 2000).

Prion proteins also bind Mn ions (Brown et al., 2000; Brown, 2001), albeit at a lower affinity, at the same octapeptide repeats that bind Cu ions. An imbalance in Cu and Mn that allows for Mn ions to replace Cu on the octapeptide repeats may impair the function of PrP^c as an antioxidant molecule (Lehmann, 2002; Deloncle et al., 2006). Furthermore, PrP^c binding Mn in place of Cu is less stable, thus allowing the protein to misfold (Brown, 2001), incur partial proteinase resistance (Brown et al., 2000), and aggregate into fibrils (Tsenkova et al., 2004). These findings are especially intriguing in light of the fact that brain tissue from TSE infected animals and humans commonly have elevated Mn and decreased Cu (Wong et al., 2001a; Wong et al., 2001b; Thackray et al., 2002).

Most research in prion biology, including that mentioned above, has been conducted *in vitro* or using rodent models. Moreover, outside of post-mortem observations in infected animals, most BSE research has also been

conducted using rodents. We previously reported no relationship between Cu status and bovine PrP^c biology (Legleiter et al., 2006); however, to our knowledge no research has been conducted to evaluate the effects of Cu status and dietary Mn in the bovine on brain metal ion concentrations and the biochemical properties of prion proteins. It is imperative that research be conducted using the bovine to verify previous studies using *in vitro* techniques and rodent models.

Thus, the objectives of this study were to determine the effect of imbalances in dietary Cu and Mn on brain Cu and Mn concentrations and on prion protein biochemical characteristics.

Materials and Methods

Animals and Experimental Design

Twelve mature Angus cows (6.1 ± 0.7 yr, 640.9 ± 28.4 kg) were used in this study. 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. Initial plasma Cu was similar for all animals so the cows were randomly assigned (4 cows per treatment) to one of three treatments: 1) control (adequate in Cu and Mn), 2) Cu-deficient (-Cu), and 3)

Cu-deficient plus high dietary Mn (-Cu+Mn). Supplemental Cu was provided from $Cu_2(OH)_3Cl$ (Micronutrients, Indianapolis, IN), Mn from $MnSO_4 \cdot H_2O$ (Sulfamex, Veracruz, Mexico), and Mo from $NaMoO_4$ (Eastern Minerals, Inc., Henderson, NC). Molybdenum, a strong Cu antagonist, was used in conjunction with a low Cu diet to induce Cu deficiency (Suttle, 1991).

The twelve multiparous cows began the study approximately 60-90 d prepartum. Calves were weaned at approximately 180 d of age. Cows were grazed in treatment groups on tall fescue pastures and were systematically rotated through pastures to minimize any pasture effects. During the winter months the cows were grazed on stockpiled tall fescue and supplemented with corn silage. The tall fescue pastures averaged 7.6 mg Cu/kg DM and 77.7 mg Mn/kg DM and the corn silage averaged 5.9 mg Cu/kg DM and 44.4 mg Mn/kg DM.

The Cu-deficient treatments received 75 mg $Mo \cdot hd^{-1} \cdot d^{-1}$ in 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 Cu/kg DM and 2,000 mg Mn/kg DM in the mineral. Based on free choice mineral consumption, control cows consumed approximately 130 mg supplemental $Cu \cdot hd^{-1} \cdot d^{-1}$ and 260 mg supplemental $Mn \cdot hd^{-1} \cdot d^{-1}$, which equates to 11.1 mg Cu/kg DM and 22.2 mg Mn/kg DM, assuming an average intake of 2% of BW. These daily intakes of Cu and Mn are adequate to meet the recommended

requirements (NRC, 1996). The -Cu treatment contained 500 mg Mo/kg DM and 2,000 mg Mn/kg DM. Daily consumption by this treatment group averaged 240 mg supplemental Mn•hd^{-1}•d^{-1} or 18.9 mg Mn/kg DM, and 60 mg supplemental Mo•hd^{-1}•d^{-1} or 4.7 mg Mo/kg DM. The -Cu+Mn treatment contained 500 mg Mo/kg DM and 50,000 mg Mn/kg DM. Consumption by this treatment group averaged 6,000 mg supplemental Mn•hd^{-1}•d^{-1} or 483 mg Mn/kg DM, and 60 mg supplemental Mo•hd^{-1}•d^{-1} or 4.8 mg Mo/kg DM. From d 120 to d 360 the cows received a daily corn gluten feed supplement (1 kg•hd^{-1}•d^{-1}) that provided the Mo (30 mg•hd^{-1}•d^{-1}) to treatments -Cu and -Cu+Mn. Daily Mo supplementation more effectively depleted liver Cu stores. During this time the cows continued to receive supplemental Cu (control) and Mn via the free choice mineral.

Liver biopsies were obtained, as described by Tiffany et al. (2003), on d 30, 120, 240, and 300 for the determination of liver Cu and Mn concentrations. On d 0, 30, 120, 240, and 300, 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.

After receiving the treatments for 360 d the cows were euthanized for the acquisition of brain tissue for prion analysis. A liver sample (approximately 100 g) was collected, transported on dry ice, and frozen (-20ºC) until analysis. The obex portion (Figure 1) 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 using the spoon technique (USDA, 2004). The obex was transported on dry ice and stored (-80ºC) until analysis.

Analytical Procedures

Liver, brain, and feed samples used for the analysis of Cu and Mn were prepared using a microwave digestion (Mars 5, CEM Corp., Matthews, NC) procedure described by Gengelbach et al. (1994). Prior to 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 (2006). Briefly, plasma was diluted 1:3 (volume:volume) in 5% trace mineral grade nitric acid (Fisher Scientific, Fair Lawn, NJ), centrifuged at 1,200 x g for 20 min, and analyzed for Cu using acetylene flame atomic absorption spectrophotometry (GFA-6500, Shimadzu Scientific Instruments, Kyoto, Japan).

Total protein was extracted from brain tissue as described by Wong et al. (2000). Approximately 1 g of chilled obex tissue was homogenized on ice in 9 mL of chilled extraction buffer (0.01 M PBS, 1% Nonidet P40, 10% w/v complete EDTA-free protease inhibitor cocktail tablets; Roche Diagnostics, Indianapolis, IN) with a Polytron (Brinkmann Instruments, Westbury, NY)

homogenizer in a 50 mL polycarbonate tube. Homogenates were immediately centrifuged at 5,000 x g for 20 minutes at 4ºC. The clarified supernatant was analyzed for total protein 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 -80ºC until analysis.

All electrophoresis and Western Blot (WB) supplies were purchased from Invitrogen Corp. (Carlsbad, CA) unless otherwise stated. Polyacrylamide Gel Electrophoresis (PAGE) was performed using pre-cast NuPAGE Novex 10% Bis-Tris gels and the Novex X-Cell Surelock Mini-Cell electrophoresis system. Magic Mark XP Western Protein Standard molecular weight marker was used for molecular weight (MW) determination. To serve as positive and negative controls, recombinant PrP^c ($rPrP^c$) ab753 (Abcam Inc., Cambridge, MA) and water, respectively, were treated exactly as samples. Proteins were separated on gels under denaturing conditions using MOPS (3-(*N*-morpholino) propane sulfonic acid) SDS (sodium dodecyl sulphate) running buffer (pH 7.3-7.7) at 200V constant for 50 minutes. After electrophoresis the gel was gently removed from the casing and the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane using the XCell II Blot Module and NuPAGE Transfer Buffer with 10% methanol and 0.1% antioxidant at a constant 30V for 60 minutes. The WB was visualized using the Western Breeze Chemiluminescent Kit. All washes were done at room temperature using a

shaker platform. Membranes were blocked and then exposed to 1:10,000 diluted anti-PrP^c mAb 6H4 (Prionics AG, Switzerland) for 1 h. After rinsing with antibody wash three times, the membrane was incubated with the secondary antibody, anti-mouse conjugated to alkaline phosphatase, for 30 minutes. The membrane was thoroughly washed and then exposed to 2.5 mL of chemiluminescent substrate (CDP-Star) for 5 minutes. To capture the WB image, Kodak X-OMAT LS film (Eastman Kodak Co., Rochester, NY) was exposed to the membrane for 4 minutes in a dark room and was subsequently developed using an auto-developer (Kodak X-OMAT Clinic 1 Processor, Eastman Kodak Co., Rochester, NY).

Membranes were then stripped using Restore Western Blot Stripping Buffer (Pierce Biotechnology, Inc., Rockford, IL) and probed for β-actin using anti-β-actin mAb AC-74 (Sigma-Aldrich, Inc., St. Louis, MO). Membranes were visualized using chemiluminescence as previously described. Beta-actin was used as an internal loading control to normalize all lanes within a gel.

The WB images were analyzed using Image Quant TL (Amersham Biosciences, Piscataway, NJ). Analysis included band identification, MW determination based on standardized molecular weight markers, and relative optical densitometry for each glycoform of PrP^c. All WB analysis was based on two to three WB replicates.

To determine the effects of treatment on prion proteinase degradability, samples were first exposed to proteinase K (PK; Bio-Rad Laboratories, Inc.,

Hercules, CA) as described by Thackray et al. (2002) prior to PAGE and WB. Briefly, brain extracts were digested with 250 ug PK/mL for 1 h at 37ºC. 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), where samples were exposed to 0, 2, 10, and 25 ug PK/mL of 10% brain tissue homogenate for 30 and 60 minutes followed by PAGE and WB, 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 and reducing agent and heating to 70ºC. Proteinase degradability was determined by comparing the WB elution profiles of PrP^c 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.

PrP^c was quantitated using a double antibody sandwich enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical Co., Ann Arbor, MI). Brain tissue homogenates (100 uL) were incubated in duplicate wells on a 96 well plate pre-coated with anti-PrP^c mouse monoclonal antibody for 2 h at room temperature. A standard curve was constructed using known quantities of $rPrP^c$ ab753. The secondary antibody, conjugated to acetylcholinesterase, was added and incubated for 2 h followed by the addition of substrate (Ellman's reagent). Color formation was allowed to develop for 30 minutes in darkness at room temperature. Absorbances were read at 405 nm using a plate reader

(Synergy HT, Bio-Tek Instruments, Inc., Winooski, VT). Ellman's reagent was used as the blank and absorbances were corrected accordingly.

Total superoxide dismutase (SOD) activity of the brain tissue homogenates was measured using a SOD kit (Cayman Chemical Co., Ann Arbor, MI). Purified SOD was used to construct a standard curve for sample SOD activity quantification. Ten microliters of protein equilibrated brain tissue homogenate was added to duplicate wells in addition to 200 uL of the radical detector (tetrazolium salt). Addition of 20 uL of xanthine oxidase to each well and incubation for 20 minutes allowed for the formation of superoxide radicals and subsequent color formation. Absorbances were read at 450 nm using a plate reader (Synergy HT, Bio-Tek Instruments, Inc., Winooski, VT). One unit (U) of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. Assays were performed in duplicate and SOD activities expressed as U/mg protein.

In addition to total SOD activity, Cu/Zn SOD and Mn SOD activities of the brain tissue homogenates were determined using the same assay. Potassium cyanide (KCN; 2mM) was used to inhibit Cu/Zn SOD in the brain tissue homogenates which subsequently allowed for the detection of Mn SOD activity. Copper/Zinc SOD activity was then calculated by the subtraction of Mn SOD activity from total SOD activity (Brown and Besinger, 1998).

Prion proteins from all brain tissue homogenates were purified using immunoprecipitation similar to that described by Brown et al. (1999). The mAb

6H4 was coupled to protein G-agarose (Sigma-Aldrich, Inc., St. Louis, MO) and subsequently mixed with brain tissue homogenates overnight at 4ºC in microtube spin columns. The beads were extensively washed and proteins subsequently eluted from the beads with the addition of 50 mM glycine (pH 4.0) and neutralized with 100 mM Tris-HCl (pH 8.0). The protein concentrations of the purified PrP^c eluates were determined using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA). The purity of the immunopurified prion eluates was confirmed by elution on polyacrylamide gels followed by Coomassie blue staining and the presence of PrP^c was confirmed via dot blotting. The SOD-like activity of immunopurified PrP^c was determined as described for brain tissue homogenates. Immunopurified PrP^c eluates were also analyzed for Cu and Mn concentrations using flameless atomic absorption spectrophotometry (GFA-6500, Shimadzu Scientific Instruments, Kyoto, Japan).

Statistical Analysis

All data was analyzed as a completely randomized design using Proc MIXED in SAS (SAS Inst. Inc., Cary, NC). In the model treatment served as the fixed effect and animal was used as a random variable. Treatment means were separated using two pre-planned single degree of freedom orthogonal contrasts: 1) control vs. -Cu and -Cu+Mn; and 2) -Cu vs. -Cu+Mn. Effects were considered significant at $P < 0.05$.

Results

Cattle receiving treatments -Cu and -Cu+Mn had lower liver Cu concentrations than controls ($P < 0.001$) on d 120, 240, and 300, as determined by liver biopsy (data not shown). Liver samples collected post-mortem indicated that cattle receiving -Cu and -Cu+Mn treatments had significantly lower liver Cu stores than control animals ($P = 0.001$; Table 1). Both treatments designed to induce Cu deficiency (-Cu and -Cu+Mn) resulted in liver Cu stores of less than 20 mg/kg DM and would be considered Cu-deficient (Underwood, 1981). Plasma Cu on d 300 averaged 0.49 ug/mL for the Cu-deficient cows, which was lower ($P = 0.001$) than control cows at 0.92 ug/mL.

The induced Cu deficiency in treatments -Cu and -Cu+Mn tended to decrease brain Cu, both obex ($P = 0.09$) and cerebellum ($P = 0.10$), relative to cows receiving adequate Cu (Table 1). Further, Cu-deficient cows (-Cu) tended ($P = 0.06$) to have lower obex Cu than Cu-deficient animals receiving high dietary Mn (-Cu+Mn; Table 1).

Liver Mn concentrations at the end of the study were similar across treatments (Table 1). Obex Mn concentrations in -Cu+Mn cattle averaged 1.6 mg/kg DM which tended to be higher ($P = 0.09$) than the 1.4 mg/kg DM in -Cu cattle (Table 1). Cerebellum Mn followed a similar pattern.

Because the obex region of the bovine brain is optimal for BSE detection and prion analysis (USDA, 2004), all data pertaining to brain prions are from obex samples. Brain prion protein concentrations were evaluated using both densitometric analysis of WB for relative comparisons and an ELISA for quantification. Densitometric analysis of WB indicated that total prion protein band relative optical densities were similar across all treatments (Figure 2). Brain prion protein concentrations, as determined by ELISA, were similar across treatments and averaged 1.98, 2.23, and 2.08 ng/g of wet tissue for the control, -Cu, and -Cu+Mn treatments, respectively (Figure 3). Thus, based on both ELISA quantified and WB relative optical densities, PrP^c concentrations were not affected by Cu deficiency or Cu-deficiency in combination with high dietary Mn.

Further analysis of WB showed that the glycoform relative distributions and the estimated MW of each glycoform were not affected by treatment (Table 2). Additionally, the banding patterns of PrP^c from all brain tissue homogenates appeared to be similar when visually inspected (Figure 4).

Exposure to 250 ug PK/mL resulted in complete degradation of all prion proteins across all treatments as indicated by the inability to detect immunoreactive prion bands in PK treated lanes on the WB versus normal detection of PrP^c in untreated samples on the same WB (Figure 5). Alternatively, BSE positive samples exposed to PK would have had PrP

fragments clearly visible on the WB as lower MW bands (Wong et al., 2001a; Deloncle et al., 2006).

To more sensitively test the effects of dietary Cu and Mn on prion proteinase degradability, the samples were exposed to lower levels of PK (0, 2, 10, 25 ug PK/mL 10% brain tissue homogenate), such that minimal changes in prion PK degradability could be detected. Thirty minutes was equally as effective as 60 minutes in degrading the prion proteins. All prion proteins were completely degraded when exposed to 10 and 25 ug PK/mL, while 2 ug PK/mL allowed for some PrP^c to remain intact and visible on the WB (Figure 6). However, this visible band was present in all samples; likely due to a very low and less than saturable PK enzyme concentration. Both PK tests indicate that dietary Cu and Mn levels did not affect PrP^c proteinase degradability and that animals were BSE negative.

Brain (obex) tissue Mn SOD activity was higher ($P = 0.05$) for -Cu and -Cu+Mn compared to animals receiving adequate Cu (Table 3). Manganese SOD in brain tissue was further increased ($P = 0.04$) in Cu-deficient cows receiving high dietary Mn (-Cu+Mn) compared to those that were only Cu-deficient (-Cu). However, total and Cu/Zn SOD activities of brain tissue homogenates were not affected by treatment. The immunopurified PrP^c eluates from the brain tissue homogenates had similar SOD-like activities (Table 3) and did not differ in Cu concentration (Figure 7). Manganese was detectable at low

levels (<1 ng/g) in some immunopurified PrP^c eluates, but most samples had undetectable levels of Mn (data not shown).

Discussion

The treatments designed to induce Cu deficiency were effective as indicated by liver and plasma Cu concentrations. By inducing a Cu deficiency in these cattle it appears that brain, particularly obex, Cu may have been decreased. In a related study (Legleiter et al., 2006) brain Cu was decreased by approximately 44% in Cu-deficient cows, whereas brain Cu in the present study only decreased 26% in Cu-deficient cows compared to Cu-adequate cows. However, the Cu-adequate cows in the present study had two to three-fold higher brain Cu concentrations than Cu-adequate cows previously tested. In the previous study all cows began the trial in a Cu-deficient state which was followed by a repletion phase where half of the cows were repleted to a Cu-adequate state while the rest remained Cu-deficient. It appears that brain Cu had not completely recovered form the Cu deficiency in the Cu-repleted cows, thus explaining the discrepancy in relative brain Cu depressions in Cu-deficient cows between the two studies.

Liver Mn concentrations were not affected by the -Cu+Mn treatment, likely due to the low absorption of dietary Mn (Hurley and Keen, 1987) and

effective homeostatic mechanisms in Mn metabolism. Homeostatic regulation of Mn in the body is primarily a function of increased hepatobiliary excretion, which can increase up to 200-fold in cattle in response to Mn loading (Hall and Symonds, 1981). Further, feeding high levels of Mn may also decrease intestinal absorption (Abrams et al., 1977). A recent study did demonstrate linear increases in liver and longissimus muscle Mn concentrations in cattle fed supplemental Mn ranging from 0 to 240 mg/kg DM (Legleiter et al., 2005); however, these were young growing cattle and the absolute increases in liver and muscle Mn appeared to have minimal biological significance. Even though liver Mn concentrations did not change in the present study, brain Mn concentrations did tend to increase in cattle receiving high dietary Mn.

While brain Cu and Mn did tend to be affected by the diet, the magnitude of these brain metal changes was much less than those seen in TSE affected tissues (Thackray et al., 2000; Wong et al., 2001a). Thus, it may not be possible to induce changes in brain Cu and Mn of the magnitude seen in TSE affected tissues; rather, those severe brain metal perturbations may be a result of the progressive disease rather than a causative factor.

There is substantial evidence linking Cu to prion biology (Brown et al., 1997; Wong et al., 2000; Kramer et al., 2001) and compelling data supporting a relationship between brain metal ion perturbations and TSE. The observational data (Purdey, 2000; Wong et al., 2001a; Thackray et al., 2002) and results from controlled experiments using rodent models and *in vitro* techniques (Brown et

al., 2000; Tsenkova et al., 2004; Deloncle et al., 2006) implicate a Mn for Cu replacement on PrP^c in the pathogenesis of TSE. This theory may be particularly relevant to sporadic TSE. However, until now the relevancy of these findings has not been tested in the bovine. The present study utilizing mature cows indicates that Cu deficiency and Cu deficiency coupled with high dietary Mn had minimal effects on the biochemical properties of PrP^c. Most notably, all prion proteins were equally and fully degradable by PK, indicating brain Cu and Mn perturbations did not induce a PrP^c to PrP^{sc} conversion, the hallmark change in all TSE.

Brain Cu and Mn concentrations also had no effect on immunopurified PrP^c SOD-like activity. However, the purported SOD-like activity of PrP^c has been questioned, as the protein was found to have minimal, if any, SOD activity both *in vitro* (Jones et al., 2005) and *in vivo* (Hutter et al., 2003). In fact, Jones et al. (2005) found PrP^c to have SOD-like activities of < 20 U/mg, which is less than 2% of the level of authentic SOD. This is similar to the PrP^c SOD-like activities report here, indicating that the activities we detected would have minimal biological significance.

The concentrations of PrP^c were similar across all treatments. Waggoner et al. (2000) demonstrated that mice expressing 0, 1, and 10 times the normal level of PrP^c had similar brain Cu concentrations and cuproenzyme activates. Taken together these findings question the importance of PrP^c in brain Cu metabolism. There were also no detectable changes in

immunopurified PrP^c Cu and Mn concentrations. The Cu concentration in the immunopurified PrP^c precipitates equates to a molar ratio of 0.25, or 0.25 atoms of Cu per molecule of PrP^c. Further, Mn was undetectable in the immunopurified PrP^c precipitates, which agrees with a recent study demonstrating PrP^c binds Cu but not Mn (Garnett and Viles, 2003). While there is intense disagreement regarding PrP^c and its ability to bind metal ions, if PrP^c does lack the ability to bind Mn, then the hypothesis tested here would indeed be rejected. Overall, these data do not support the hypothesis that an imbalance in brain Cu and Mn results in altered prion protein biochemical characteristics.

Future research investigating the relationship between Cu, Mn, and brain prions in the bovine should consider the effects of length and severity of brain metal imbalances. Although brain Cu and Mn appeared to be altered in cows exposed to low Cu diets and low Cu diets coupled with high dietary Mn, the magnitude of the perturbations in brain Cu and Mn may not have been great enough to effectively alter PrP^c biology. While the liver Cu concentrations of cows assigned to the Cu-deficient treatments were well below 20 mg/kg DM, which was indicative of severe Cu deficiency, their average plasma Cu concentration of 0.49 ug/mL on d 300 suggests they may have been only marginally deficient. Thus, a more severe Cu deficiency may depress brain Cu even further than seen in this study. Likewise, a more significant increase in brain Mn than seen in this study may be required to affect prion biology. While

the respiratory and gastrointestinal tracts are the primary means of entry into the body, most Mn toxicities in humans are associated with inhalation (Oberdoester and Cherian, 1988), primarily because inhaled Mn is more likely to reach the central nervous system before hepatic clearance (Heilig et al, 2005). In rats, administering $MnCl_2$ via oral gavage and intraperitoneal injection had no effect on cerebellum and striatum Mn concentrations, but intratracheal instillation significantly increased Mn in both tissues (Roels et al., 1997). Although little is known about the effects of Mn inhalation in the bovine, this route of exposure may produce much higher brain Mn concentrations that could have deleterious effects on PrP^c. Finally, the cows in this study were exposed to Cu deficiency and high dietary Mn for approximately one year with minimal effects on brain prion proteins. Prolonged exposure to these imbalances in Cu and Mn may bring forth different results.

We have previously demonstrated in the bovine that Cu status appears to have minimal effects on brain PrP^c (Legleiter et al., 2006); however, to our knowledge this is the first study investigating the relationships between dietary Cu and Mn and brain prion protein characteristics using the bovine as a model. We conclude that altering brain Cu and Mn by exposing mature cows to low Cu diets, or low Cu diets plus high dietary Mn, for one year had minimal effects on prion protein biology.

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Table 1. Effect of dietary Cu and Mn levels on liver and brain Cu and Mn concentrations[a]

	Treatments				Contrasts[b]	
	Control	-Cu	-Cu+Mn	SEM	Control vs. -Cu & -Cu+Mn	-Cu vs. -Cu+Mn
					-- P values --	
Copper, mg/kg DM						
Liver	264.1	7.5	15.6	9.7	0.001	0.57
Obex	22.8	12.9	20.6	2.6	0.09	0.06
Cerebellum	27.0	18.8	19.1	3.6	0.10	0.95
Manganese, mg/kg DM						
Liver	14.1	13.5	14.7	1.0	0.97	0.44
Obex	1.5	1.4	1.6	0.07	0.92	0.09
Cerebellum	2.7	2.4	2.9	0.2	0.89	0.17

[a]Samples collected following euthanasia after 360 d on study.

[b]Pre-planned single degree of freedom contrast used to separate the Least Squares Means.

Table 2. Effect of dietary Cu and Mn levels on prion protein molecular weights and relative glycoform distributions[a,b]

	Treatments				Contrasts[c]	
	Control	-Cu	-Cu+Mn	SEM	Control vs. -Cu & -Cu+Mn	-Cu vs. -Cu+Mn
Molecular weight, kD[d]					*-- P values --*	
Diglycosylated	36.0	36.3	36.2	0.1	0.10	0.52
Monoglycosylated	32.3	32.4	32.4	0.1	0.27	0.70
Unglycosylated	28.1	28.4	28.2	0.2	0.29	0.29
Glycoform distribution, %[e]						
Diglycosylated	35.8	36.3	37.5	1.4	0.54	0.56
Monoglycosylated	23.7	23.1	22.4	1.0	0.46	0.65
Unglycosylated	40.5	40.6	40.1	1.1	0.94	0.75

[a]PrP^c has two glycosylation sites allowing for the presence of three glycoforms.

[b]Reported means from the analysis of two to three Western blots.

[c]Pre-planned single degree of freedom contrast used to separate the Least Squares Means.

[d]Estimated based on gel migrations relative to a standardized molecular weight marker using gel analysis software (ImageQuant TL, Amersham Bioscience).

[e]Estimated based on the relative optical density of each band relative to all immunoreactive PrP^c from that sample.

Table 3. Effect of dietary Cu and Mn levels on brain tissue homogenate and immunopurified prion superoxide dismutase (SOD) activities[a]

	Treatments				Contrasts[b]	
	Control	-Cu	-Cu+Mn	SEM	Control vs. -Cu & -Cu+Mn	-Cu vs. -Cu+Mn
Brain (Obex)					*-- P values --*	
Total SOD, U[c]/mg[d]	60.7	64.8	79.1	7.6	0.26	0.21
Cu/Zn SOD, U/mg	47.2	49.2	57.3	6.2	0.44	0.37
Mn SOD, U/mg	13.5	15.6	21.8	1.9	0.05	0.04
Prion protein						
SOD, U/mg[e]	47.8	65.3	53.9	9.7	0.35	0.43

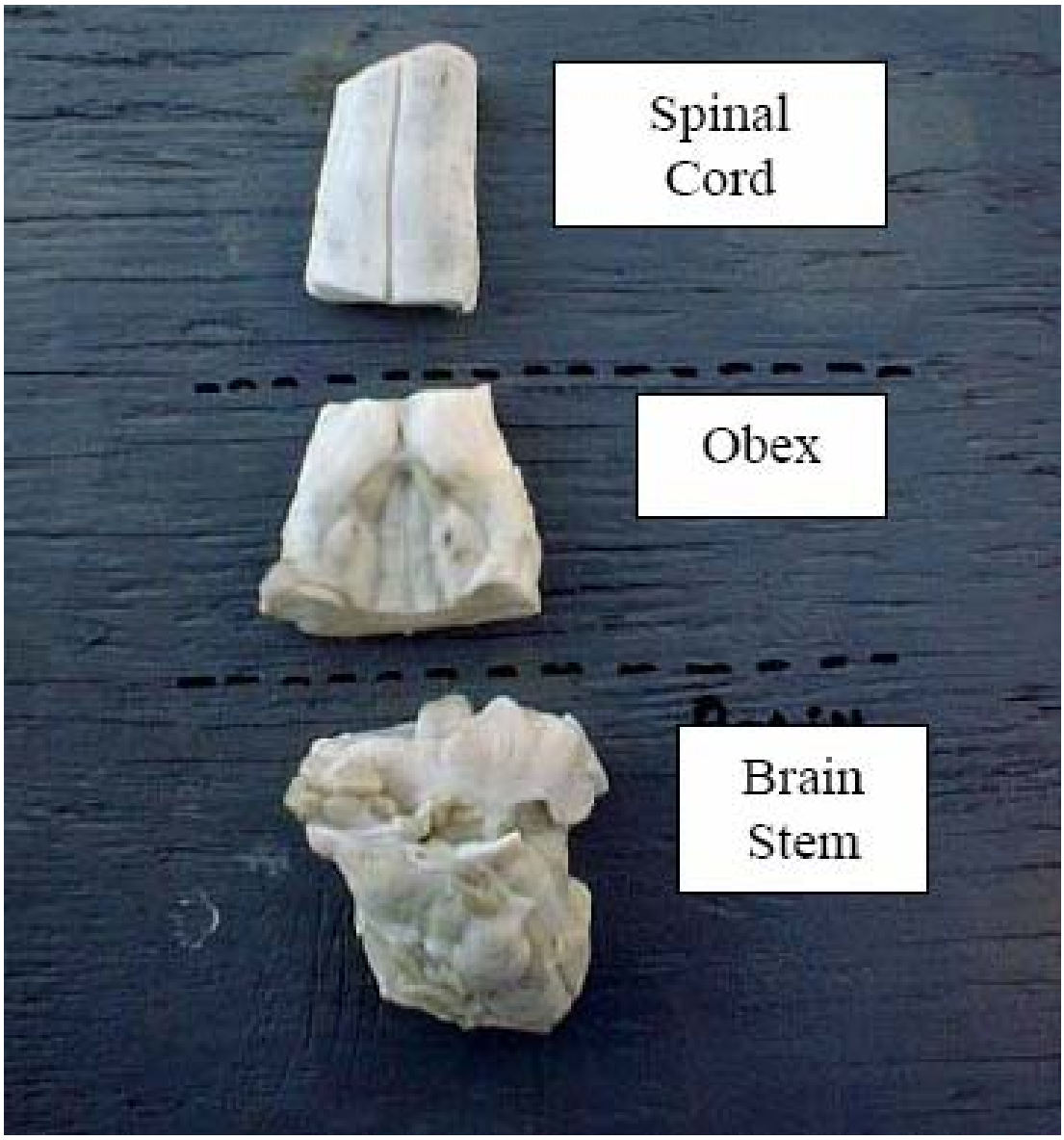
[a]All assays performed in duplicate or triplicate.

[b]Pre-planned single degree of freedom contrast used to separate the Least Squares Means.

[c]One unit (U) is equal to the activity required for 50% dismutation of the superoxide radicals.

[d]Expressed as U/mg of protein in the 10% brain tissue homogenate.

[e]Expressed as U/mg of protein in the immunopurified PrP^c eluate.



Source: USDA, 2004

Figure 1. The obex portion of the bovine brain stem. The obex was removed through the occipital foramen using the spoon technique (USDA, 2004). This is the primary tissue used for BSE surveillance and diagnosis, therefore it was used for all prion analysis reported here.

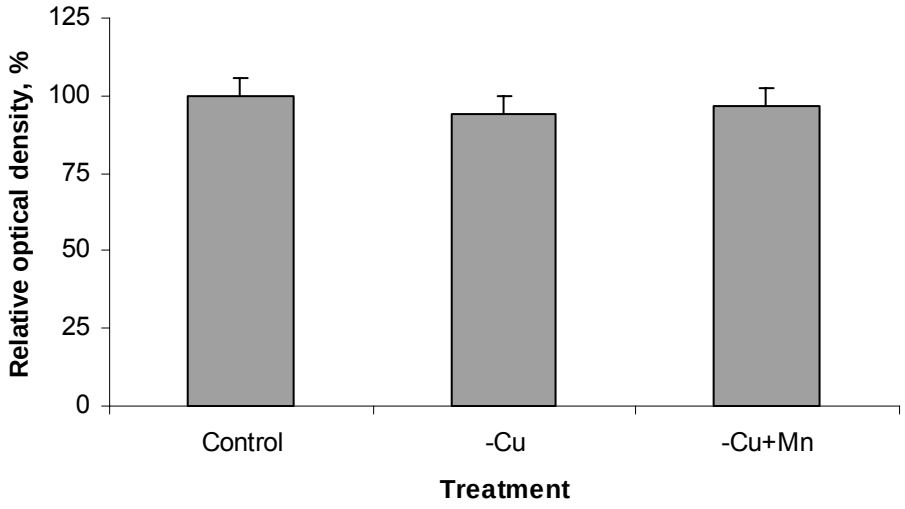


Figure 2. Effects of dietary Cu and Mn levels on relative concentrations of immunoreactive prion proteins. The relative optical densities of immunoreactive brain (obex) prion protein bands were determined by densitometric analysis of Western blots. Optical densities of -Cu and -Cu+Mn immunoreactive prion bands are expressed as a percent of the control. Means are based on the analysis of two to three Western blots.

control vs. -Cu and -Cu+Mn, $P = 0.50$

-Cu vs. -Cu+Mn, $P = 0.78$

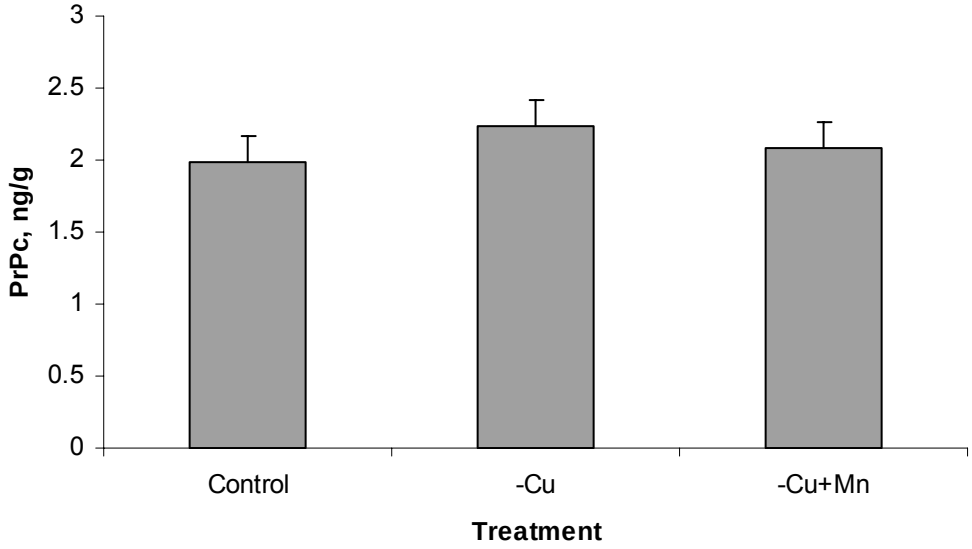


Figure 3. Effects of dietary Cu and Mn levels on brain prion protein concentrations. Brain (obex) prion protein (PrP^c) concentrations were determined by enzyme-linked immunsorbent assay using a standard curve constructed from known quantities of recombinant PrP^c. Means are expressed as ng PrP^c/g obex tissue.

control vs. -Cu and -Cu+Mn, $P = 0.44$

-Cu vs. -Cu+Mn, $P = 0.56$

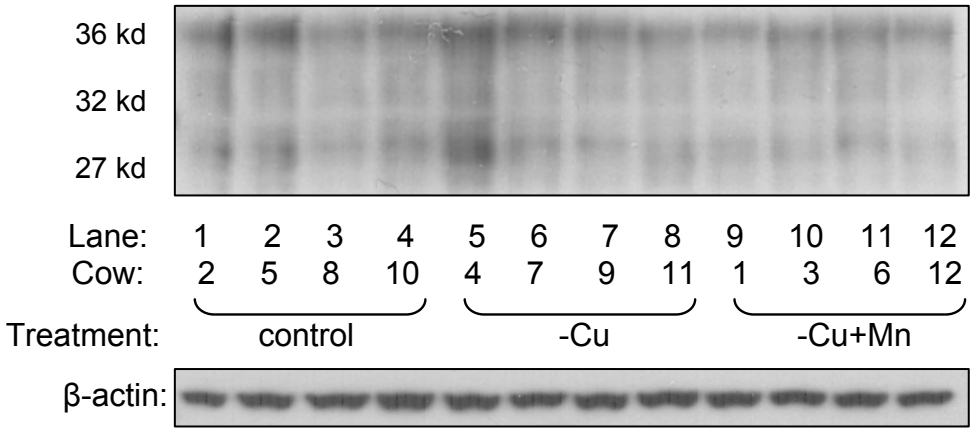


Figure 4. Effects of dietary Cu and Mn levels on prion protein elution profiles. This representative Western blot shows immunoreactive prion proteins (PrP^c) from brain tissue homogenates from all twelve cows. Densitometric analysis indicated that relative optical densities of PrP^c bands were similar across treatments. Further, glycoform distributions and molecular weights were not affected by treatment. Visual analysis of the blot shows similar elution profiles across treatments. Treatments: control; Cu-deficient (-Cu); and Cu-deficient plus high dietary Mn (-Cu+Mn). β-actin was used to normalize all lanes.

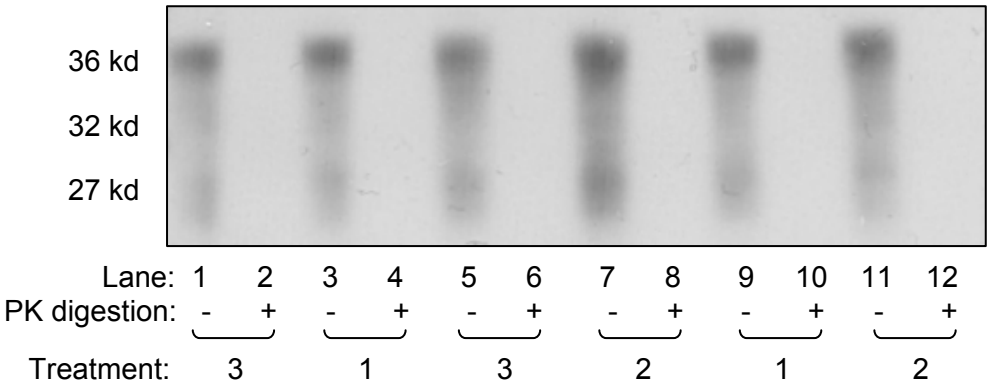


Figure 5. Effects of dietary Cu and Mn levels on prion protein proteinase degradability. A representative western blot (WB) of prion proteins from brain tissue homogenates with proteinase K (PK) exposed (250 ug PK/mL) and unexposed samples run in parallel. All prion proteins from each animal and across all treatments that were exposed to PK were completely degraded. Treatments: 1) Cu-adequate (control); 2) Cu-deficient (-Cu); 3) Cu-deficient plus high dietary Mn (-Cu+Mn). Treatment did not affect PrP^c PK degradability as all PrP^c was completely degraded in brain tissue homogenates from all cows.

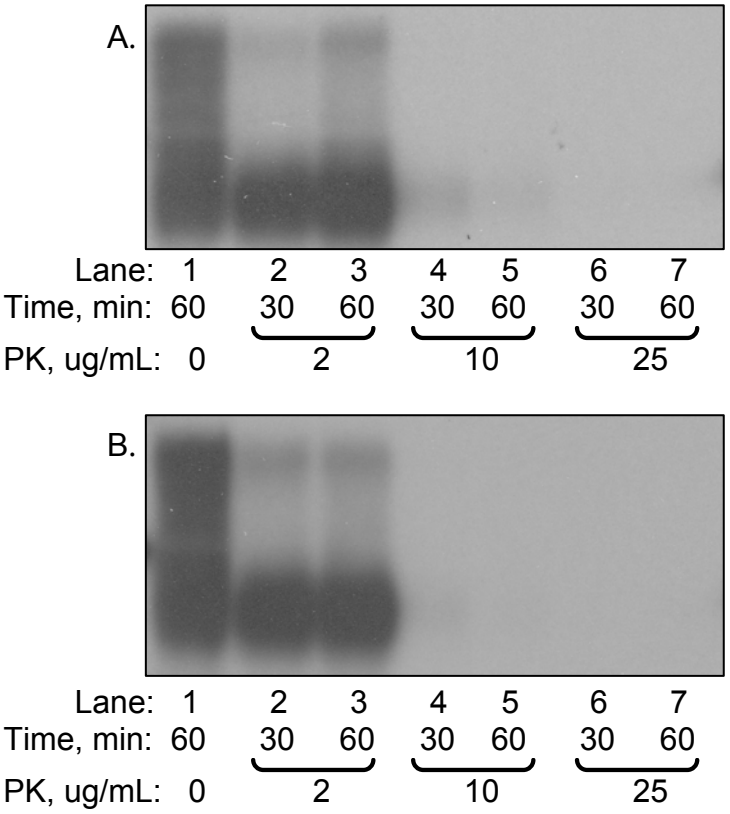


Figure 6. Effects of dietary Cu and Mn levels on prion protein degradability by proteinase K. Representative western blots (two samples) of prion proteins from brain tissue homogenates exposed to 0, 2, 10, and 25 ug PK/mL 10% brain tissue homogenate for 30 and 60 minutes. For both blots (A & B) shown here, as well as all other samples tested, 10 and 25 ug PK/mL 10% brain tissue homogenate completely degraded all prion proteins while 2 ug PK/mL 10% brain tissue homogenate degraded only a portion of the prion proteins in the sample. Prion proteins from all animals across all treatments were degraded in a similar manner.

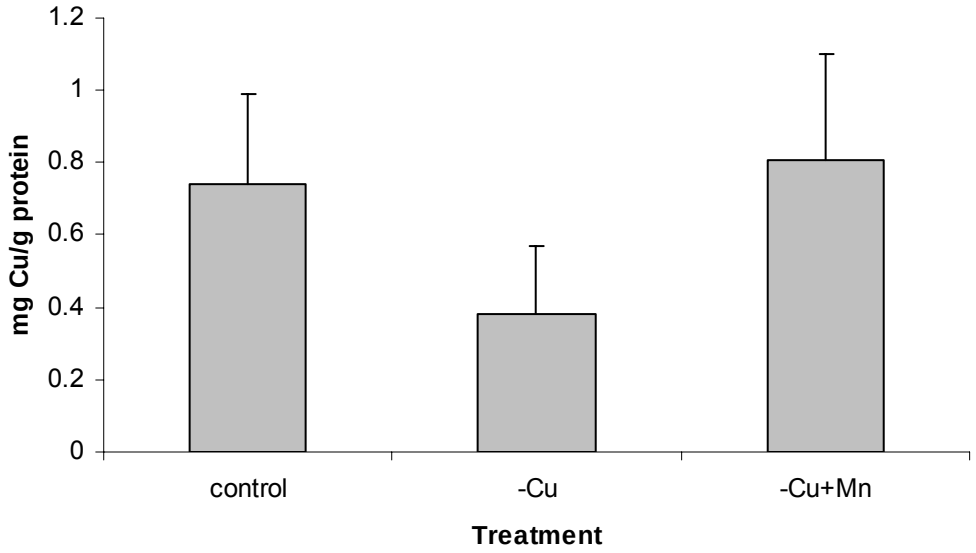


Figure 7. Effects of dietary Cu and Mn levels on immunopurified prion protein Cu content. The copper concentrations of immunopurified prion protein eluates, as determined by flameless atomic absorption spectrophotometry, were not affected by treatment. Means are expressed as mg Cu/g protein.
control vs. -Cu and -Cu+Mn, $P = 0.65$
-Cu vs. -Cu+Mn, $P = 0.29$

CHAPTER 4

Long-term exposure to low dietary copper or low dietary copper coupled with excess dietary manganese induces brain metal perturbations but does not significantly alter brain prion protein characteristics in the mature bovine[1,2]

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[1]Use of trade names in this publication does not imply endorsement by the North Carolina Agric. Research Serv. or criticism of similar products not mentioned.

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Abstract

Recent findings from a one year study using mature cows failed to support the hypothesis that an imbalance in brain copper (Cu) and manganese (Mn) results in altered brain prion biochemical characteristics. The present study further tests this hypothesis by exposing twelve mature cows to low Cu diets or low Cu diets coupled with high dietary Mn for 20 months to determine effects on brain Cu and Mn concentrations and prion biochemical properties. Copper-adequate cows were randomly assigned to treatments: 1) control (adequate in Cu and Mn), 2) Cu-deficient (-Cu), and 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 molybdenum (Mo) to further induce Cu deficiency. After 600 d Cu-deficient cows (-Cu & -Cu+Mn) had decreased brain Cu (P = 0.003) and increased brain Mn (P = 0.05) relative to controls. Immunopurified prion eluates had similar Cu concentrations. According to Western blot analysis, prion relative optical densities, proteinase K degradability, elution profiles, and glycoform distributions were similar across treatments. The concentration of prion, as determined by ELISA, was not affected by treatment. Brain tissue antioxidant capacity and superoxide dismutase (SOD) activities were not compromised by perturbations in brain metals. Immunopurified prion had similar SOD-like activities among treatments. Although brain copper was decreased and Mn increased due to exposure to low

Cu diets with and without high Mn, the brain metal imbalance had no effect on brain antioxidant status and PrP^c functional characteristics.

Key Words: Bovine, Copper, Manganese, Prion

Introduction

Transmissible spongiform encephalopathies (TSE) are a class of neurodegenerative diseases that includes Creutzfeldt-Jakob disease (CJD), scrapie, chronic wasting disease (CWD) and bovine spongiform encephalopathy (BSE; Prusiner, 1995). These diseases manifest as genetic, infectious, or sporadic disorders; all of which result from the misfolding of the cellular prion protein (PrP^{c}) to the infective and disease causing isoform (PrP^{sc}; Prusiner, 2004) that is partially resistant to proteinase K (PK) degradation (Prusiner et al., 1998).

There continues to be a growing interest in the role of metal ions, particularly copper (Cu) and manganese (Mn), in TSE (Brown, 2001; Lehmann, 2002). Prions cooperatively bind Cu ions (Brown et al., 1997; Brown, 1999; Kramer et al., 2001) resulting in a stabilized structure (Hornshaw et al., 1995) and an acquired Cu-dependent SOD-like activity (Brown et al., 1997; Brown et al., 1999; Wong et al., 2000).

Manganese can also be incorporated into native PrP^{c} via cooperative binding at the same octapeptide repeats that bind Cu ions (Brown et al., 2000; Brown, 2001). Large increases in brain Mn coupled with decreases in brain Cu have been associated with TSE (Wong et al., 2001a; Wong et al., 2001b;

Thackray et al., 2002). An imbalance in Cu and Mn that allows for Mn ions to replace Cu on the octapeptide repeats may impair the function of PrP^c as an antioxidant molecule (Lehmann, 2002; Deloncle et al., 2006) and allow for structural changes (Brown, 2001) resulting in proteinase resistant prions (Brown et al., 2000; Deloncle et al., 2006). As proposed by Sulkowski (1992), these findings implicate metal ions in the pathogenesis of prion diseases, particularly sporadic TSE.

The hypothesis that brain Cu and Mn perturbations are responsible for key changes in prion characteristics is largely based on *in vitro* techniques and rodent model-based work. Feeding mature cows a diet low in Cu (Legleiter et al., 2006a) or low in Cu and high in Mn (Legleiter et al., 2006b) for one year altered brain Cu and Mn concentrations but had minimal effects on brain prion protein properties. Copper deficiency may have to exist for an extended period of time to deplete Cu from brain prions. The present study was conducted to determine if a longer period of Cu deficiency, alone or coupled with high dietary Mn would more dramatically alter brain Cu and Mn concentrations and prion protein biochemical characteristics.

Materials and Methods

Animals and Experimental Design

Twelve mature Angus cows (5.7 ± 0.8 yr, 628.0 ± 34.2 kg) were used in this study. 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. Initial plasma Cu was similar for all animals so the cows were randomly assigned (4 cows per treatment) to one of three treatments: 1) control (adequate for Cu and Mn), 2) Cu-deficient (-Cu), and 3) Cu-deficient plus high dietary Mn (-Cu+Mn). Supplemental Cu was provided from $Cu_2(OH)_3Cl$ (Micronutrients, Indianapolis, IN), Mn from $MnSO_4 \cdot H_2O$ (Sulfamex, Veracruz, Mexico), and Mo from $NaMoO_4$ (Eastern Minerals, Inc., Henderson, NC). Supplemental Mo was used to induce Cu deficiency (Suttle, 1991).

The twelve multiparous cows were maintained on the study through two calving seasons. The gestating cows began the study approximately 60 to 90 d prepartum and the calves born during that first year were weaned on d 270. The cows calved the subsequent year between d 410 and d 450 of the study. The second-year calves were weaned on d 600, at which point the study ended.

Cows were grazed in treatment groups on tall fescue pastures and were systematically rotated through pastures to minimize any pasture effects. During

the winter months the cows were grazed on stockpiled tall fescue and supplemented with corn silage. The tall fescue pastures averaged 7.4 mg Cu/kg DM and 74.1 mg Mn/kg DM and the corn silage averaged 5.3 mg Cu/kg DM and 38.7 mg Mn/kg DM. The Cu-deficient treatments received 75 mg $Mo \cdot hd^{-1} \cdot d^{-1}$ in 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 Cu/kg DM and 2,000 mg Mn/kg DM. Based on free choice mineral consumption, control cows consumed 130 mg supplemental $Cu \cdot hd^{-1} \cdot d^{-1}$ and 260 mg supplemental $Mn \cdot hd^{-1} \cdot d^{-1}$, which equates to 10.3 mg Cu/kg DM and 20.6 mg Mn/kg DM, assuming an average intake of 2% of BW. These daily intakes of Cu and Mn are adequate to meet the recommended requirements (NRC, 1996). The -Cu treatment contained 500 mg Mo/kg DM and 2,000 mg Mn/kg DM. Consumption by this treatment group averaged 240 mg supplemental $Mn \cdot hd^{-1} \cdot d^{-1}$ or 20.3 mg Mn/kg DM, and 60 mg supplemental $Mo \cdot hd^{-1} \cdot d^{-1}$ or 5.1 mg Mo/kg DM. The -Cu+Mn treatment contained 500 mg Mo/kg DM and 50,000 mg Mn/kg DM. Consumption by this treatment group averaged 6,000 mg supplemental $Mn \cdot hd^{-1} \cdot d^{-1}$ or 531 mg Mn/kg DM, and 60 mg supplemental $Mo \cdot hd^{-1} \cdot d^{-1}$ or 5.3 mg Mo/kg DM. From d 120 to d 540 the cows received a daily corn gluten feed supplement (1 $kg \cdot hd^{-1} \cdot d^{-1}$) that provided the Mo (30 $mg \cdot hd^{-1} \cdot d^{-1}$) to treatments -Cu and -Cu+Mn. Daily Mo supplementation more effectively depleted liver Cu stores. During this time the cows continued

to receive supplemental Cu (control) and Mn via the free choice mineral. From d 540 to 600 the cows were housed in covered slotted floor pens with outside runs. They were bunk fed a corn-silage based diet (11.5% CP, 69% TDN, 19.2 mg Cu/kg DM, 48.8 mg Mn/kg DM) and remained on their respective treatments with regard to supplemental Cu, Mn, and Mo. The control diet was formulated to provide 10 mg supplemental Cu/kg DM and 20 mg supplemental Mn/kg DM. Treatment -Cu provided 20 mg supplemental Mn/kg DM and 2 mg supplemental Mo/kg DM while -Cu+Mn provided 500 mg supplemental Mn/kg DM and 2 mg supplemental Mo/kg DM.

Liver biopsies were obtained, as described by Tiffany et al. (2003), on d 30, 120, 240, 300, 390 and 540 for the determination of liver Cu and Mn concentrations. On the same days jugular blood samples were collected into heparinized (Vacutainer 9735, Becton Dickinson, Franklin Lakes, NJ) tubes specifically designed for trace mineral analysis for plasma Cu determination.

After receiving the treatments for 20 mo cows were transported approximately 60 km to an abattoir and slaughtered. A liver sample (approximately 100 g) was collected, transported on dry ice, and frozen (-20ºC) 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 using the spoon technique (USDA, 2004). The obex was transported on dry ice and stored (-80ºC) until analysis.

Analytical Procedures

Liver, brain, and feed samples used for the analysis of Cu and Mn were prepared using a microwave digestion (Mars 5, CEM Corp., Matthews, NC) procedure described by Gengelbach et al. (1994). Prior to 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 (2006). Briefly, plasma was diluted 1:3 (volume:volume) in 5% trace mineral grade nitric acid (Fisher Scientific, Fair Lawn, NJ), centrifuged at 1,200 x g for 20 min, and analyzed for Cu using acetylene flame atomic absorption spectrophotometry (GFA-6500, Shimadzu Scientific Instruments, Kyoto, Japan).

Total protein was extracted from brain tissue as described by Wong et al. (2000). Approximately 1 g of chilled obex tissue was homogenized on ice in 9 mL of chilled extraction buffer (0.01 M PBS, 1% Nonidet P40, 10% w/v complete EDTA-free protease inhibitor cocktail tablets; Roche Diagnostics, Indianapolis, IN) with a Polytron (Brinkmann Instruments, Westbury, NY) homogenizer in a 50 mL polycarbonate tube. Homogenates were immediately centrifuged at 5,000 x g for 20 minutes at 4ºC. The clarified supernatant was analyzed for total protein 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 -80ºC until analysis.

All electrophoresis and Western Blot (WB) supplies were purchased from Invitrogen Corp. (Carlsbad, CA) unless otherwise stated. Polyacrylamide Gel Electrophoresis (PAGE) was performed using pre-cast NuPAGE Novex 10% Bis-Tris gels and the Novex X-Cell Surelock Mini-Cell electrophoresis system. Magic Mark XP Western Protein Standard molecular weight marker was used for molecular weight (MW) estimation. Recombinant PrP^c ($rPrP^c$) ab753 (Abcam Inc., Cambridge, MA) and water were used as positive and negative controls, respectively.

Proteins were separated on gels under denaturing conditions using MOPS (3-(*N*-morpholino) propane sulfonic acid) SDS (sodium dodecyl sulphate) running buffer (pH 7.3-7.7) at 200V constant for 50 minutes. After electrophoresis the gel was gently removed from the casing and the proteins transferred onto a polyvinylidene difluoride (PVDF) membrane using the XCell II Blot Module and NuPAGE Transfer Buffer with 10% methanol and 0.1% antioxidant at a constant 30V for 60 minutes. The WB was visualized using the Western Breeze Chemiluminescent Kit. All washes were done at room temperature using a shaker platform. After blocking to reduce non-specific binding, the membrane was exposed to 1:10,000 diluted anti-PrP^c mAb 6H4 (Prionics AG, Switzerland) for one h. After rinsing with antibody wash three

times, the membrane was incubated with the secondary antibody, anti-mouse conjugated to alkaline phosphatase, for 30 minutes. The membrane was then thoroughly rinsed and exposed to 2.5 mL of chemiluminescent substrate (CDP-Star) for 5 minutes. To capture the WB image, Kodak X-OMAT LS film (Eastman Kodak Co., Rochester, NY) was exposed to the membrane for 4 minutes in a dark room and was subsequently developed using an auto-developer (Kodak X-OMAT Clinic 1 Processor, Eastman Kodak Co., Rochester, NY).

Membranes were then stripped using Restore Western Blot Stripping Buffer (Pierce Biotechnology, Inc., Rockford, IL) and probed for β-actin using anti-β-actin mAb AC-74 (Sigma-Aldrich, Inc., St. Louis, MO). Membranes were visualized using chemiluminescence as previously described. Beta-actin was used as an internal loading control to normalize all lanes within a gel.

The WB images were analyzed using Image Quant TL (Amersham Biosciences, Piscataway, NJ). Analysis included band identification, MW estimation, and relative optical densitometry for each glycoform of PrP^c. The PrP^c has two glycosylation sites allowing for three glycoforms: unglycosylated, monoglycosylated, and diglycosylated PrP^c. The identification and densitometric analysis of all 3 glycoforms allowed for the calculation of glycoform distribution as a percent of total PrP^c. Further, densitometric analysis of all three glycoforms allowed PrP^c relative optical densities to be calculated for

each treatment and expressed as a percent of the control treatment. All WB analysis was based on two to three WB replicates.

To determine the effects of treatment on prion proteinase degradability, samples were first exposed to proteinase K (PK; Bio-Rad Laboratories, Inc., Hercules, CA) as described by Thackray et al. (2002) prior to PAGE and WB. Briefly, 40 ul of 10% brain tissue homogenate was mixed with 250 ug PK/mL in a microcentrifuge tube and allowed to digest for one h at 37ºC. 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 ug PK/mL of 10% brain tissue homogenate, was used to more sensitively test the effects of brain Cu and Mn on the proteinase degradability of prion proteins. For both PK tests, the reaction was stopped with the addition of loading buffer and reducing agent and heating to 70ºC. Proteinase degradability was determined by comparing the WB elution profiles of PrP^c 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.

PrP^c was quantitated using a double antibody sandwich enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical Co., Ann Arbor, MI). Brain tissue homogenates were incubated in duplicate wells on a 96 well plate pre-coated with anti-PrP^c mouse monoclonal antibody for 2 h at room temperature. A standard curve was constructed using known quantities of

rPrP^{c} ab753. Wells were rinsed with wash buffer five times followed by addition of the second anti-PrP^{c} antibody conjugated to acetylcholinesterase and incubation for 2 h. After thoroughly washing all wells with wash buffer, 200 ul of Ellman's Reagent was added to each well and incubated for 30 minutes in darkness at room temperature. Absorbances were read at 405 nm using a plate reader (Synergy HT, Bio-Tek Instruments, Inc., Winooski, VT). Ellman's reagent was used as the blank and absorbances were corrected accordingly.

Total superoxide dismutase (SOD) activity of the brain tissue homogenates was measured using a kit (Cayman Chemical Co., Ann Arbor, MI). Purified SOD was used to construct a standard curve for sample SOD activity quantification. Ten microliters of protein equilibrated brain tissue homogenate was added to duplicate wells in addition to 200 ul of the radical detector (tetrazolium salt). Addition of 20 ul of xanthine oxidase to each well and incubation for 20 minutes allowed for the formation of superoxide radicals and subsequent color formation. Absorbances were read at 450 nm using a plate reader (Synergy HT, Bio-Tek Instruments, Inc., Winooski, VT). One unit (U) of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. Assays were performed in duplicate and SOD activities expressed as U/mg protein.

In addition to total SOD activity, Cu/Zn SOD and Mn SOD activities of the brain tissue homogenates were determined using the same assay. Potassium cyanide (KCN; 2mM) was used to inhibit Cu/Zn SOD in the brain tissue

homogenates, which subsequently allowed for the detection of Mn SOD activity. Copper/Zn SOD activity was then calculated by the subtraction of Mn SOD activity from total SOD activity (Brown and Besinger, 1998).

Brain tissue homogenates were also assayed for their overall antioxidant capacity (Cayman Chemical Co., Ann Arbor, MI). The assay relies on the ability of antioxidants in the sample to inhibit the oxidation of 2,2'-Azino-di-(3-ethylbenzthiazoline sulphonate) by metmyoglobin. The suppression of the absorbance, measured at 750 nm, is proportional to the concentration of the antioxidants which is expressed relative to Trolox, a water-soluble tocopherol analogue. The assay was performed in duplicate.

Lipid peroxidation was measured in brain tissue homogenates by assaying for lipid hydroperoxides (LPO) using the lipid hydroperoxide assay kit (Cayman Chemical Co., Ann Arbor, MI). Lipid hydroperoxides are detected by their ability to oxidize ferrous ions. The resulting ferric ions are subsequently detected using thiocyanate as a chromagen, which can be monitored by measuring the absorbance at 500 nm.

Prion proteins from all brain tissue homogenates were purified using immunoprecipitation similar to that described by Brown et al. (1999). The mAb 6H4 was coupled to protein G-agarose (Sigma-Aldrich, Inc., St. Louis, MO) and subsequently mixed with brain tissue homogenates overnight at 4ºC in microtube spin columns. The beads were extensively washed and proteins subsequently eluted from the beads with the addition of 50 mM glycine (pH 4.0)

and neutralized with 100 mM Tris-HCl (pH 8.0). The protein concentrations of the purified PrP^c eluates were determined using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA). The purity of the immunopurified prion eluates was confirmed by elution on polyacrylamide gels followed by Coomassie blue staining and the presence of PrP^c was confirmed via dot blotting. The SOD-like activity of immunopurified PrP^c was determined as described for brain tissue homogenates. Immunopurified PrP^c eluates were also analyzed for Cu and Mn concentrations using flameless atomic absorption spectrophotometry (GFA-6500, Shimadzu Scientific Instruments, Kyoto, Japan).

Statistical Analysis

All data was analyzed using Proc MIXED in SAS (SAS Inst. Inc., Cary, NC). Liver Cu and Mn values collected over the duration of the study were analyzed as repeated measures with fixed effects of treatment, time, and treatment x time. Animal served as a random effect. When the treatment x time interaction was significant ($P < 0.05$) each time point was analyzed separately to further define the interaction. When the interaction was not significant it was removed from the model. All post-mortem brain and liver data as well as all prion data were analyzed as a completely randomized design with animal serving as the experimental unit. For all analyses treatment means were separated using two pre-planned single degree of freedom orthogonal

contrasts: 1) control vs. -Cu and -Cu+Mn; and 2) -Cu vs. -Cu+Mn. Effects were considered significant at $P < 0.05$.

Results

When analyzed as repeated measures, treatment ($P < 0.001$) and d ($P = 0.004$) significantly affected liver Cu concentrations (Figure 1). Liver Cu concentrations in -Cu and -Cu+Mn cows were below values indicative of Cu deficiency (20 mg Cu/kg DM; Underwood, 1981) by at least d 300 and remained low throughout the remainder of the study. Ending liver Cu concentrations were lower in treatments -Cu and -Cu+Mn relative to the control group ($P = 0.001$; Table 1). On d 540 plasma Cu concentrations were much higher ($P = 0.001$; 1.25 ug/dL) in controls compared to cows in the -Cu (0.17 ug/mL) and -Cu+Mn (0.13 ug/mL) treatments.

The induced Cu deficiency in treatments -Cu and -Cu+Mn decreased brain (obex) Cu ($P = 0.003$) relative to cattle receiving adequate Cu (Table 1). Cattle receiving -Cu+Mn tended ($P = 0.09$) to have lower obex Cu than -Cu animals.

When analyzed as repeated measures liver Mn was similar across all treatments over the duration of the study (Figure 2). Ending liver Mn tended ($P = 0.09$) to be higher in Cu-deficient cattle (-Cu and -Cu+Mn) compared to controls (Table 1). Obex Mn concentrations were increased in Cu-deficient

cattle compared to Cu-adequate cattle (P = 0.05). However, obex Mn concentrations did not differ among -Cu and -Cu+Mn treatments.

Densitometric analysis of WB indicated that PrP^c band relative optical densities were similar across all treatments (Figure 3). Brain prion protein concentrations, as determined by ELISA, were also similar among treatments (Figure 4). Thus, based on both ELISA quantified and WB relative optical densities, PrP^c concentrations were not affected by Cu deficiency or Cu deficiency in combination with high dietary Mn.

The relative distribution and estimated MW of the three glycoforms were similar across the three treatments (Table 2). Additionally, based on visual analysis there were no apparent differences in the banding patterns of PrP^c from all brain tissue homogenates (Figure 5). Exposure to 250 ug PK/mL resulted in complete degradation of all prion proteins across all treatments as indicated by the inability to detect immunoreactive prion bands in PK treated lanes on the WB versus normal detection of PrP^c in untreated samples on the same WB (Figure 5). All prion proteins were completely degraded when exposed to 10 and 25 ug PK/mL, while 2 ug PK/mL allowed for some PrP^c to remain intact and visible on the WB (Figure 6). However, this visible band was present in all samples; likely due to a very low and less than saturable PK enzyme concentration. Both PK tests indicate Cu and Mn did not affect PrP^c proteinase degradability and that all cattle were BSE negative.

Brain (obex) tissue SOD activities (total, Cu/Zn, and Mn SOD) were not affected by treatment (Table 3). Likewise, immunopurified PrP^c had similar SOD-like activity across treatments. The overall antioxidant capacity of brain tissue homogenates was similar across all treatments indicating brain metal perturbations did not compromise the antioxidant defense systems (Figure 7). Lipid hydroperoxides, resulting from lipid peroxidation, were assayed in brain tissue homogenates but all values were below the detection limit (0.25 nmol hydroperoxide) of the assay (data not shown).

The immunopurified PrP^c eluates did not differ in Cu concentration (Figure 8). Manganese was detectable at low levels (<1 ug/g protein) in some immunopurified PrP^c eluates, but most samples had undetectable levels of Mn (data not shown).

Discussion

This study demonstrates that brain metal perturbations in mature cows induced by exposure to low Cu diets, alone or coupled with high dietary Mn, for twenty mo does not significantly alter brain prion protein characteristics. These results agree with previously reported studies (Legleiter et al., 2006a; Legleiter et al., 2006b). Results from these studies are in contrast to compelling observational (Purdey, 2000; Thackray et al., 2002) and experimental evidence

(Wong et al., 2001a; Brown et. al., 2000; Deloncle et al., 2006) linking Cu and Mn to biochemical changes in PrP^c. However, this and our previous studies differ in that the bovine was used as a model, versus rodents or *in vitro* methodologies.

Exposing the cows on treatments -Cu and -Cu+Mn to low Cu diets supplemented with Mo for 20 mo induced a severe Cu deficiency as indicated by liver and plasma Cu concentrations. Cows in the present study had much lower ending plasma Cu concentrations than cows described by Legleiter et al. (2006b). This coupled with liver Cu concentrations well below 20 mg/kg DM indicates these cows were more severely Cu-deficient than cows in the previous study. Additionally, the cows in the present study were maintained in a Cu-deficient state for approximately 300 d, which is twice as long as the previous study. Unlike previously reported (Legleiter et al., 2006b), liver Mn tended to be affected by treatment. However, it was unexpectedly increased in -Cu+Mn cows as well as cows receiving treatment -Cu relative to controls.

The severe Cu deficiency decreased brain Cu 31% and 58%, for treatments -Cu and -Cu+Mn, respectively. Similar to liver Mn, brain Mn concentrations were increased, approximately 20%, in -Cu and -Cu+Mn treatments compared to controls. The increased severity of the Cu deficiency coupled with the extended exposure (20 mo) to the -Cu and -Cu+Mn treatments allowed for greater changes in brain Cu and Mn than seen in cows receiving similar treatments for one year (Legleiter et al., 2006b).

Nevertheless, even with a large reduction in brain Cu and an increase in Mn, there were minimal effects on the biochemical properties of PrP^c. There were no detectable changes in immunopurified PrP^c Cu and Mn concentrations, indicating Mn did not replace PrP^c-bound Cu ions. Based on the Cu-binding analysis conducted by Brown et al. (2000) using recombinant PrP^c, the concentrations reported here equate to a Cu to PrP^c molar ratio of 0.21, or 0.21 Cu atoms per molecule of PrP^c. This is significantly less bound Cu than the proposed maximal PrP^c binding potential of 4 atoms of Cu per molecule of PrP^c (Brown et al., 2000). However, using recombinant Syrian hamster PrP^c, Stockel et al. (1998) reported a lower maximal binding capacity of 1.8 Cu atoms per PrP^c. Though caution should be exercised when extrapolating from *in vitro* to *in vivo* studies; the concentrations of Cu in the immunopurified PrP^c precipitates in this study indicates that PrP^c may indeed bind Cu *in vivo* in the bovine, but at lower concentrations than reported *in vitro* using recombinant PrP^c. Most importantly, Cu deficiency and the subsequent decrease in brain Cu did not affect the apparent PrP^c bound Cu. The inability to detect Mn in immunopurified PrP^c is in agreement with a recent study that demonstrated PrP^c binds Cu but not Mn (Garnett and Viles, 2003); which, if true *in vivo*, completely undermines the hypothesis that a Mn for Cu replacement on PrP^c is involved in TSE.

All other PrP^c characteristics were unaffected by the decrease in brain Cu and increase in Mn. Most notably, all prion proteins were equally and fully degradable by PK, indicating brain Cu and Mn perturbations did not induce a

PrP^{c} to PrP^{sc} conversion, the hallmark change in all TSE. Thus, these data do not support the hypothesis that an imbalance in brain Cu and Mn results in altered prion protein biochemical characteristics. As discussed previously (Legleiter et al., 2006b), this agrees with other researchers who have questioned the importance of PrP^{c} in Cu metabolism and antioxidant defense systems (Waggoner et al., 2000; Hutter et al., 2003; Jones et al., 2005).

The perturbed brain Cu and Mn concentrations also had no apparent effect on the antioxidant defense systems of the brain, as the total antioxidant capacity and SOD activities were not affected. This is in slight contrast to that reported by Legleiter et al. (2006b), where Mn SOD was increased in Cu-deficient cows. The Mn SOD activity in Cu-deficient animals was numerically higher in this study as well, but not statistically different. The discrepancy between the Mn SOD activities in the two studies is unclear. Nevertheless, realizing no changes in the antioxidant defense systems coupled with the lack of detectable lipid hydroperoxides in this study indicates the changes in Cu and Mn did not significantly increase oxidative stress.

Further, brain Cu and Mn concentrations also had no effect on immunopurified PrP^{c} SOD-like activity. However, as discussed previously (Legleiter et al., 2006b), the PrP^{c} SOD-like activities detected may have minimal biological significance, particularly when compared to the activity of true SOD (Hutter et al., 2003; Jones et al., 2005)

Similar to previous studies conducted in our laboratory (Legleiter et al., 2006a; Legleiter et al., 2006b), the relative decreases in brain Cu have been quite large compared to increases in brain Mn. Brain Mn may have to be increased to a greater extent to elicit changes in PrP^c as describe by others. However, it is difficult to increase both hepatic and extrahepatic Mn by feeding high levels of dietary Mn due to low absorption (Hurley and Keen, 1987) and effective homeostatic mechanisms (Abrams et al., 1977; Hall and Symonds, 1981). As discussed by Legleiter et al. (2006b), inhalation of Mn may produce significantly higher brain Mn concentrations (Roels et al., 1997; Heilig et al, 2005) that might allow for the hypothesized Mn for Cu substitution on PrP^c to occur. Future research investigating the relationships between prions, Cu, and Mn should address this issue.

This study was conducted to test the hypothesis that an imbalance in brain Cu and Mn would result in changes in the biochemical properties of brain prion proteins. Unlike most research in prion biology, the hypothesis was tested *in vivo* using the bovine as a model. We conclude that decreasing brain Cu and increasing brain Mn by exposing mature cows to low Cu diets, or low Cu diets plus high dietary Mn, for 20 mo has minimal effects on prion protein biology and brain antioxidant capacity. These conclusions are in agreement with previous reports (Legleiter et al., 2006a; Legleiter et al., 2006b).

Implications

It has been suggested that imbalances in brain Cu and Mn could initiate or increase the incidence of prion diseases, particularly sporadic forms of the diseases. This study concludes that Cu deficiency in the bovine alone or in combination with high levels of dietary Mn can alter brain Cu and Mn concentrations; however, the decreased brain Cu and increased brain Mn do not significantly affect prion protein biology. Most importantly, the imbalances in brain Cu and Mn in this study did not allow for the formation of proteinase resistant prion proteins or any other biochemical properties indicative of infective prions. Thus, if BSE can occur sporadically, a dietary imbalance in Cu and Mn does not appear to be a causative factor.

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Table 1. Effect of dietary Cu and Mn levels on liver and brain Cu and Mn concentrations[a]

	Treatments				Contrasts[b]	
	control	-Cu	-Cu+Mn	SEM	Control vs. -Cu & -Cu+Mn	-Cu vs. -Cu+Mn
					-- P values --	
Copper, mg/kg DM						
Liver	224.4	9.4	11.7	26.3	0.001	0.95
Obex	23.8	16.4	10.1	2.2	0.003	0.09
Manganese, mg/kg DM						
Liver	10.7	12.6	11.6	0.6	0.09	0.32
Obex	1.5	1.9	1.8	0.15	0.05	0.49

[a]Samples collected following euthanasia after 360 d on study.

[b]Pre-planned single degree of freedom contrast used to separate the Least Squares Means.

Table 2. Effect of dietary Cu and Mn levels on prion protein molecular weights and relative glycoform distributions[a,b]

	Treatments				Contrasts[c]	
	control	-Cu	-Cu+Mn	SEM	Control vs. -Cu & -Cu+Mn	-Cu vs. -Cu+Mn
Molecular weight, kD[d]					*-- P values --*	
Diglycosylated	35.6	35.5	36.0	0.2	0.46	0.15
Monoglycosylated	32.4	32.1	32.8	0.1	0.56	0.007
Unglycosylated	26.1	26.1	26.6	0.2	0.26	0.08
Glycoform distribution, %[e]						
Diglycosylated	16.5	19.1	18.4	1.2	0.15	0.71
Monoglycosylated	20.2	18.5	22.1	1.4	0.95	0.11
Unglycosylated	63.3	62.4	59.5	2.1	0.36	0.37

[a]PrP^c has two glycosylation sites allowing for the presence of three glycoforms.

[b]Reported means from the analysis of two to three Western blots.

[c]Pre-planned single degree of freedom contrast used to separate the Least Squares Means.

[d]Estimated based on gel migrations relative to a standardized molecular weight marker using gel analysis software (ImageQuant TL, Amersham Bioscience).

[e]Estimated based on the relative optical density of each band relative to all immunoreactive PrP^c from that sample.

Table 3. Effect of dietary Cu and Mn levels on brain tissue homogenate and immunopurified prion protein superoxide dismutase (SOD) activities[a]

	Treatments				Contrasts[b]	
	control	-Cu	-Cu+Mn	SEM	Control vs. -Cu & -Cu+Mn	-Cu vs. -Cu+Mn
Brain (obex)					*-- P values --*	
Total SOD, U[c]/mg[d]	67.5	71.9	77.7	8.0	0.44	0.64
Cu/Zn SOD, U/mg	47.6	48.5	54.1	5.2	0.55	0.49
Mn SOD, U/mg	19.9	23.4	23.6	3.0	0.32	0.96
Prion protein						
SOD, U/mg[e]	63.3	52.8	59.7	11.6	0.59	0.68

[a]All assays performed in duplicate or triplicate.

[b]Pre-planned single degree of freedom contrast used to separate the Least Squares Means.

[c]One unit (U) is equal to the activity required for 50% dismutation of the superoxide radicals.

[d]Expressed as U/mg of protein in the 10% brain tissue homogenate.

[e]Expressed as U/mg of protein in the immunopurified PrP^c eluate.

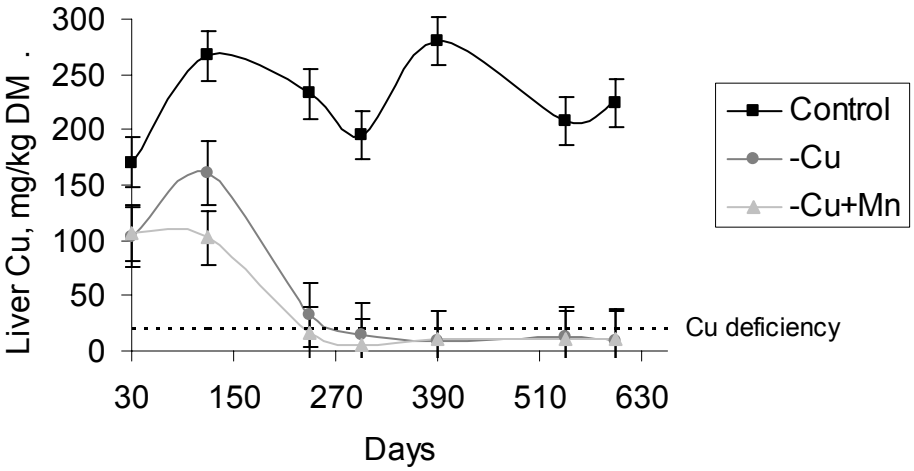

Figure 1. Liver Cu concentrations from d 30 to d 600. Analyzing the data as repeated measures using Proc MIXED in SAS revealed main effects of treatment ($P < 0.001$) and d ($P = 0.004$). Both treatments designed to induce Cu deficiency (-Cu and -Cu+Mn) resulted in lower liver Cu stores (control vs. -Cu and -Cu+Mn, $P < 0.001$) compared to control animals.

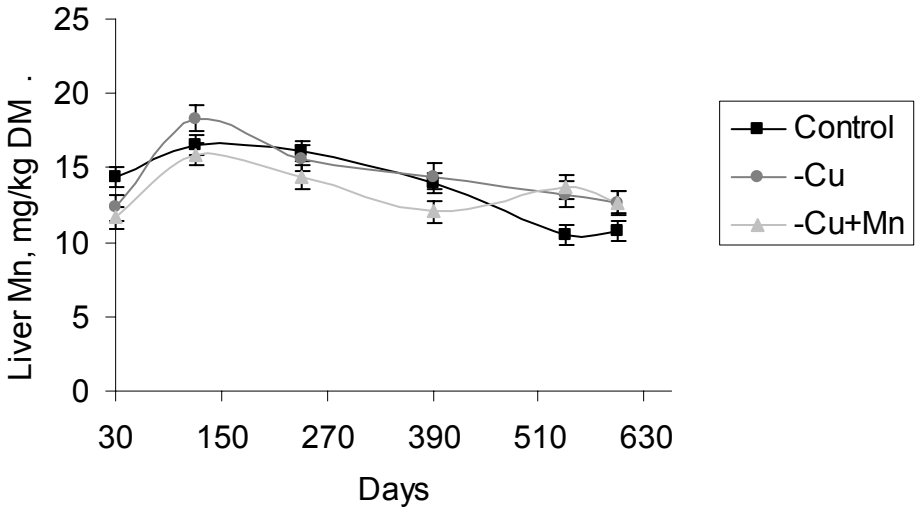

Figure 2. Liver Mn concentrations from d 30 to d 600. Treatment (P = 0.71) had no affect on liver Mn concentrations over the duration of the study.

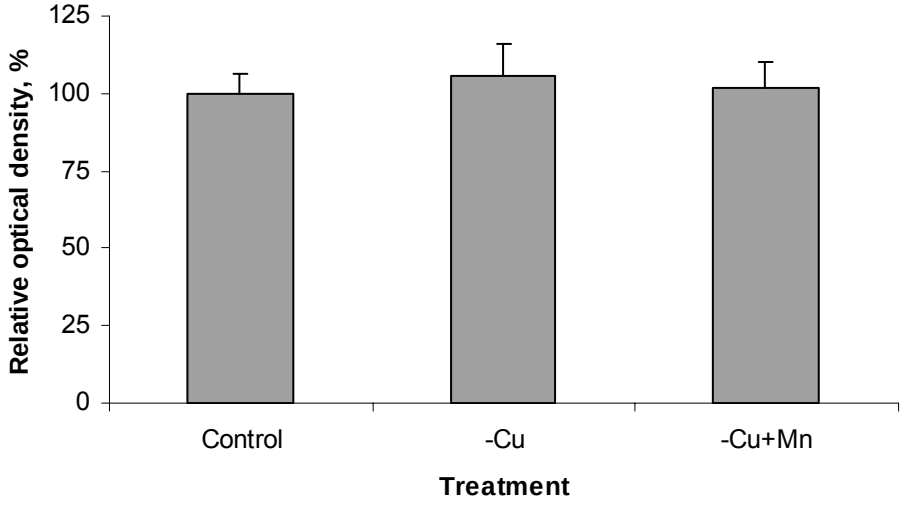

Figure 3. Effects of dietary Cu and Mn levels on relative concentrations of immunoreactive prion proteins. The relative optical densities of immunoreactive prion protein bands were determined by densitometric analysis of Western blots. Optical densities of -Cu and -Cu+Mn immunoreactive prion proteins are expressed as a percent of the control. Means are based on the analysis of two to three Western blots.

control vs. -Cu and -Cu+Mn, $P = 0.71$

-Cu vs. -Cu+Mn, $P = 0.76$

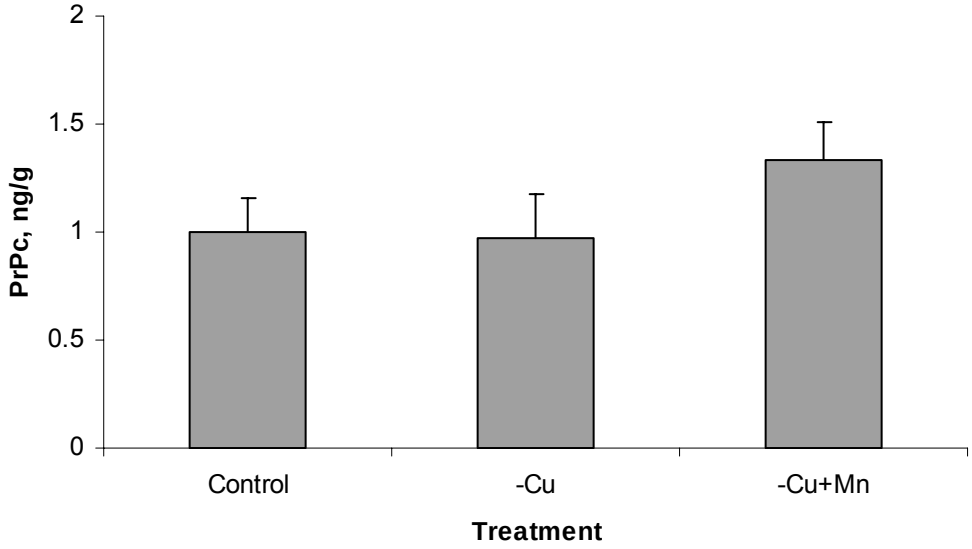

Figure 4. Effects of dietary Cu and Mn levels on brain prion protein concentrations. Brain (obex) prion protein (PrP^c) concentrations were determined by enzyme-linked immunsorbent assay using a standard curve constructed from known quantities of recombinant PrP^c. Means are expressed as ng PrP^c/g obex tissue.

control vs. -Cu and -Cu+Mn, $P = 0.52$

-Cu vs. -Cu+Mn, $P = 0.23$

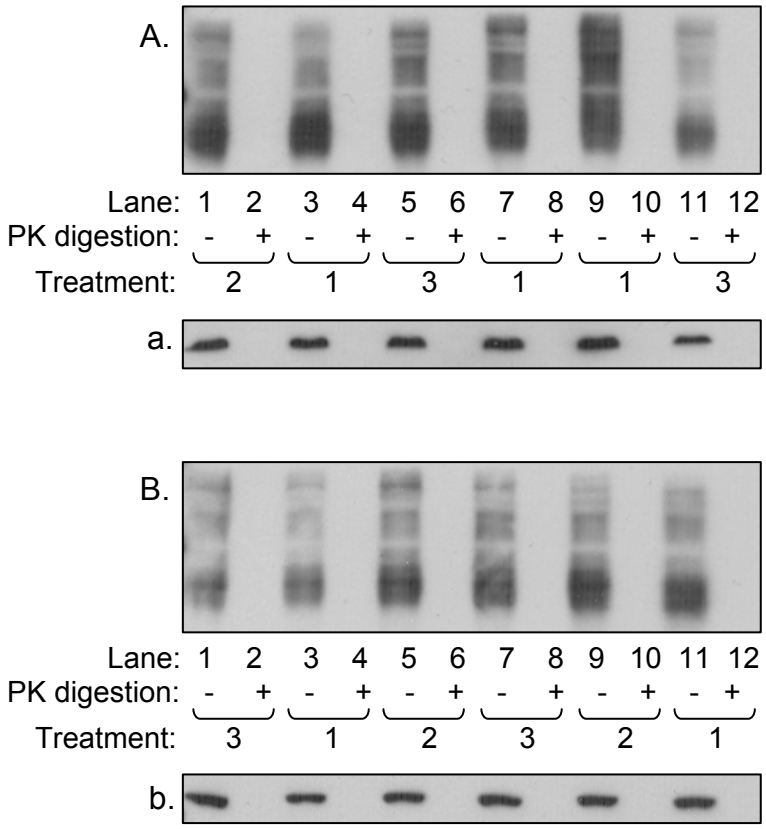

Figure 5. Effects of dietary Cu and Mn levels on the proteinase degradability and elution profiles of brain prion proteins. Western blots (WB) of prion proteins from brain tissue homogenates from all twelve cows (Panels A and B) with proteinase K (PK) exposed (250 ug PK/mL) and unexposed samples run in parallel. All prion proteins from each animal and across all treatments that were exposed to PK were completely degraded. These WB were also used to calculate prion protein molecular weights, glycoform distribution and relative optical densities. Visual analysis of the WB shows similar banding patterns across treatments. Treatments: 1) 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).

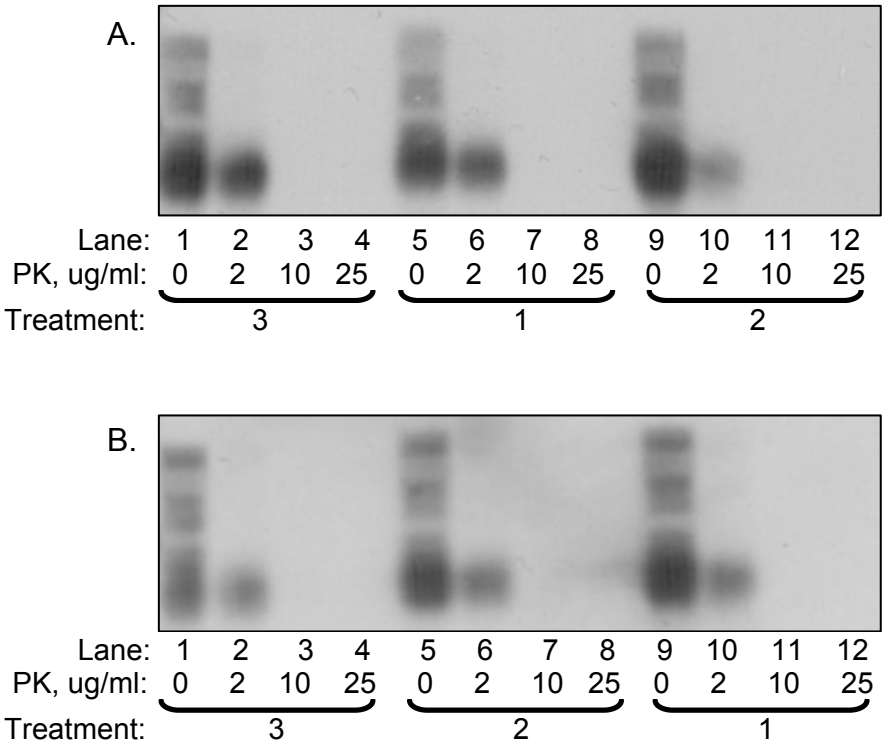

Figure 6. Effects of dietary Cu and Mn levels on prion protein degradability by proteinase K. The representative Western blots (WB) are prion proteins from brain tissue homogenates exposed to 0, 2, 10, and 25 ug proteinase K (PK)/mL 10% brain tissue homogenate for 30 minutes at 37°C. For both blots (A & B) shown here, as well as all other samples tested, 10 and 25 ug PK/mL 10% brain tissue homogenate completely degraded all prion proteins while 2 ug PK/mL 10% brain tissue homogenate degraded most but not all of the prion proteins in the sample. However, prion proteins from all animals across all treatments were degraded in a similar manner indicating treatment had no effect on PK degradability. Treatments: 1) control; 2) Cu-deficient (-Cu); 3) Cu-deficient plus high dietary Mn (-Cu+Mn).

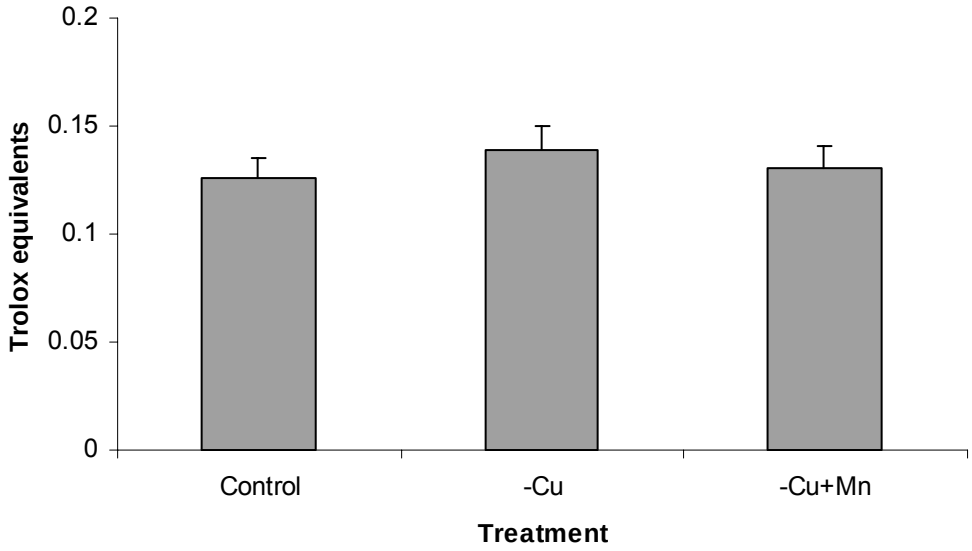

Figure 7. Effects of dietary Cu and Mn levels on the total antioxidant capacity of brain tissue homogenates. The antioxidant capacity of obex tissue homogenates was determined by the inhibition of ethylbenzthiazoline sulphonate oxidation by metmyoglobin. The antioxidant capacity is expressed as Trolox, a water-soluble tocopherol analogue, equivalents.
control vs. -Cu, $P = 0.46$
-Cu vs. -Cu+Mn, $P = 0.59$

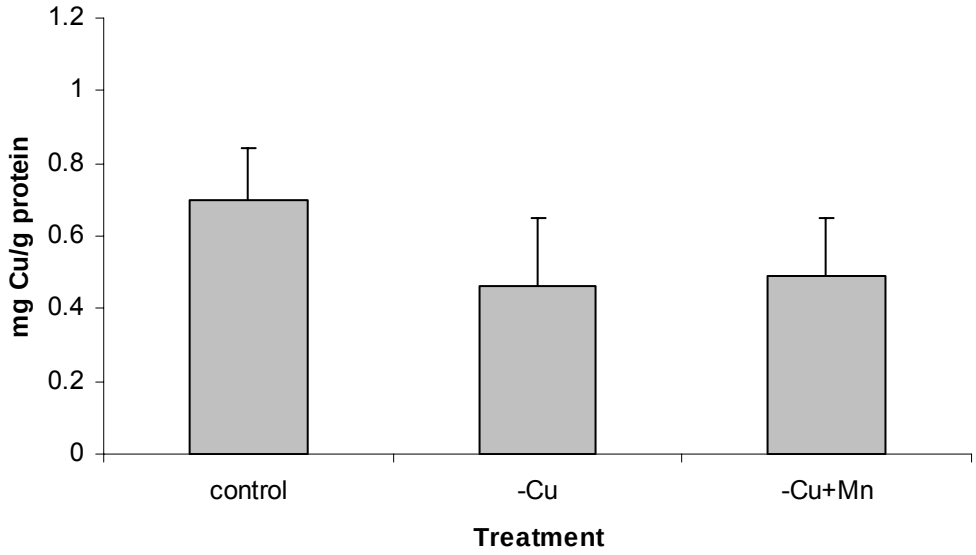

Figure 8. Effects of dietary Cu and Mn levels on immunopurified prion protein Cu content. Copper concentrations of immunopurified PrP^c eluates were determined by flameless atomic absorption spectrophotometry. Means are expressed as mg Cu/g protein.

control vs. -Cu and -Cu+Mn, $P = 0.27$

-Cu vs. -Cu+Mn, $P = 0.91$

CHAPTER 5

Copper deficiency in the young bovine results in dramatic decreases in brain copper concentrations but does not significantly alter brain prion protein biology[1,2]

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[1]Use of trade names in this publication does not imply endorsement by the North Carolina Agric. Research Serv. or criticism of similar products not mentioned.

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Abstract

A manganese (Mn) for copper (Cu) substitution on cellular prion proteins (PrP^c) in the brain that results in biochemical changes to PrP^c has been implicated in the pathogenesis of transmissible spongiform encephalopathies. Recent research in the mature bovine does not support this theory. In the present study this hypothesis was tested using progeny from gestating cows receiving low Cu diets and low Cu diets coupled with high dietary Mn. Copper-adequate cows (n=39) were randomly assigned to treatments: 1) control (adequate in Cu and Mn), 2) Cu-deficient (-Cu), and 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 molybdenum (Mo) to further induce Cu deficiency. Calves were weaned at 180 d and maintained on the same treatments as their respective dams. After 260 d on feed the cattle were harvested. Copper-deficient cattle (-Cu & -Cu+Mn) had decreased brain Cu ($P = 0.001$) and tended to have increased brain Mn ($P = 0.09$) relative to controls. Brain prion protein characteristics, including proteinase K degradability, superoxide dismutase (SOD)-like activity, and glycoform distributions were largely unaffected by treatment. Brain tissue antioxidant capacity was not compromised by perturbations in brain metals, but Cu-deficient cattle tended to have decreased ($P = 0.06$) Cu/Zn SOD activity and increased ($P = 0.06$) Mn

SOD activity. Although brain copper was decreased due to Cu deficiency and Mn increased due to exposure to high dietary Mn, the brain metal imbalance had minimal effects on PrP^c functional characteristics.

Key Words: Bovine, Copper, Manganese, Prion

Introduction

Bovine spongiform encephalopathy (BSE) is one of several fatal neurodegenerative diseases in the family of transmissible spongiform encephalopathies (TSE). The causative agent in all TSE is believed to be the pathological isoform (PrP^{sc}) of the cellular prion protein (PrP^{c}; Prusiner, 1982). The mechanism(s) responsible for the conversion of PrP^{c} to PrP^{sc} are not clear, however, the metal ions copper (Cu) and manganese (Mn) have been implicated in the pathogenesis of TSE (Brown 2001; Lehmann, 2002). The cellular prion protein cooperatively binds Cu ions (Brown et al., 1997a; Brown, 1999; Kramer et al., 2001) resulting in a stabilized structure (Hornshaw et al., 1995) and acquisition of an apparent Cu-dependent SOD-like activity (Brown et al., 1997a; Brown et al., 1999; Wong et al., 2000). Manganese may also bind to PrP^{c} (Brown et al., 2000; Brown, 2001); however, a Mn for Cu substitution on PrP^{c} may impair their function as an antioxidant molecule (Lehmann, 2002; Deloncle et al., 2006) and allow for structural changes (Brown, 2001) resulting in proteinase resistance prions (Brown et al., 2000; Deloncle et al., 2006). Thus, an imbalance in brain Cu and Mn that allows for Mn ions to replace Cu on PrP^{c} may be involved in the pathogenesis of prion diseases. This hypothesis is particularly attractive considering large increases in brain Mn coupled with decreases in brain Cu have been associated with TSE (Wong et al., 2001a; Wong et al., 2001b; Thackray et al., 2002).

Previous research conducted in this laboratory using the mature bovine (Legleiter et al., 2006a; Legleiter et al., 2006b; Legleiter et al., 2006c), as well as 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 of exposing the young bovine to low Cu diets or low Cu plus high dietary Mn on brain Cu and Mn concentrations, and on prion protein biochemical characteristics.

Materials and Methods

Animals and Experimental Design

Thirty-nine Angus cows (5.6 ± 0.5 yr, 624.8 ± 19.0 kg) and their progeny were used in this study. 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. Copper-adequate cows were randomly assigned (13 cows per treatment) to one of three treatments: 1) control (adequate in Cu and Mn), 2) Cu-deficient (-Cu), and 3) Cu-deficient plus high dietary Mn (-Cu+Mn). Supplemental Cu was provided from $Cu_2(OH)_3Cl$

(Micronutrients, Indianapolis, IN), Mn from $MnSO_4 \cdot H_2O$ (Sulfamex, Veracruz, Mexico), and Mo from $NaMoO_4$ (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 $Mo \cdot hd^{-1} \cdot d^{-1}$.

The goal was to induce Cu deficiency in gestating cows receiving treatments -Cu and -Cu+Mn, as well as 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 60-90 d prepartum and were maintained on those treatments until the calves were weaned at an average of 180 d. Cows were grazed in treatment groups on tall fescue pastures and were systematically rotated through pastures to minimize any pasture effects. During the winter months the cows were grazed on stockpiled tall fescue and supplemented with corn silage. The tall fescue pastures averaged 7.6 mg Cu/kg DM and 77.7 mg Mn/kg DM and the corn silage averaged 5.9 mg Cu/kg DM and 44.4 mg Mn/kg DM. The Cu-deficient treatments received 75 mg $Mo \cdot hd^{-1} \cdot d^{-1}$ 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 Cu/kg DM and 2,000 mg Mn/kg DM. Based on free choice mineral consumption, control cows consumed 130 mg supplemental $Cu \cdot hd^{-1} \cdot d^{-1}$ and 260 mg supplemental $Mn \cdot hd^{-1} \cdot d^{-1}$, which equates to 10.7 mg Cu/kg DM and

21.3 mg Mn/kg DM, assuming an average intake of 2% of BW. These daily intakes of Cu and Mn are adequate to meet recommended requirements (NRC, 1996). The -Cu treatment contained 500 mg Mo/kg DM and 2,000 mg Mn/kg DM. Consumption by this treatment group averaged 240 mg supplemental $Mn \cdot hd^{-1} \cdot d^{-1}$ or 20.0 mg Mn/kg DM, and 60 mg supplemental $Mo \cdot hd^{-1} \cdot d^{-1}$ or 5.0 mg Mo/kg DM. The -Cu+Mn treatment contained 500 mg Mo/kg DM and 50,000 mg Mn/kg DM. Consumption by this treatment group averaged 6,000 mg supplemental $Mn \cdot hd^{-1} \cdot d^{-1}$ or 500 mg Mn/kg DM, and 60 mg supplemental $Mo \cdot hd^{-1} \cdot d^{-1}$ or 5.0 mg Mo/kg DM. From d 120 through weaning the cows received a daily corn gluten feed supplement (1 $kg \cdot hd^{-1} \cdot d^{-1}$) that provided the Mo (30 $mg \cdot hd^{-1} \cdot d^{-1}$) to treatments -Cu and -Cu+Mn. Daily Mo supplementation more effectively depleted liver Cu stores. During this time the cows continued to receive supplemental Cu (control) and Mn 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.

Within 24 h of birth the calves were weighed, a blood sample collected for the determination of initial plasma Cu, and male calves were castrated. Blood samples were subsequently collected 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 protection against infectious bovine rhinotracheitis, bovine viral diarrhea (I and II), parainfluenza-3, bovine respiratory syncitial virus, (for protection against infectious bovine rhinotracheitis, bovine viral diarrhea (I and II), parainfluenza-3, bovine respiratory syncitial virus, (Titanium 5, AgriLabs, St. Joseph, MO), *Clostridia* (Vision 7, Intervet, Millsboro, DE), and *Moraxella bovis* (Piliguard Pinkeye-1 Trivalent, Schering-Plough Animal Health, Ltd., Wellington, New Zealand). Cattle were also treated for internal and external parasites (Privermectin, First Priority, Inc., Elgin, Illinois). 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 prior to initiation of the growing phase. During this weaning and acclimation period the calves were fed corn silage and received no supplemental Cu, Mn, or Mo. Once acclimated, each calf began receiving the same treatment that their respective dam received during gestation and lactation. The treatments were formulated to provide the following: control (10 mg Cu/kg DM, 20 mg Mn/kg DM); -Cu (20 mg Mn/kg DM, 5 mg Mo/kg DM); and -Cu+Mn (500 mg Mn/kg DM, 5 mg Mo/kg DM). The calves received a corn silage-based growing diet (Table 1; 7.0 mg Cu/kg DM, 42.7 mg Mn/kg 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 140 d on the growing diet the steers and heifers were implanted with Synovex-Plus (Fort Dodge Animal Health, Fort Dodge, IA) and gradually switched (over a 14-d period) to a corn-based finishing diet (Table 1; 5.4 mg Cu/kg DM, 17.6 mg Mn/kg DM) for 120 d. All diets were formulated to meet or exceed all nutrient requirements except for Cu (NRC, 1996). Cattle 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 frozen (-20ºC) 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 using the spoon technique (USDA, 2004). The obex was transported on dry ice and stored (-80ºC) until analysis.

Analytical Procedures

Liver, brain, and feed samples used for the analysis of Cu and Mn were prepared using a microwave digestion (Mars 5, CEM Corp., Matthews, NC) procedure described by Gengelbach et al. (1994). Prior to 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 (2006). Briefly, plasma was diluted 1:3 (volume:volume) in 5% trace mineral grade nitric acid (Fisher Scientific, Fair Lawn, NJ), centrifuged at 1,200 x g for 20 min, and analyzed for Cu using acetylene flame atomic absorption spectrophotometry (GFA-6500, Shimadzu Scientific Instruments, Kyoto, Japan).

Total protein was extracted from brain tissue as described by Wong et al. (2000). Approximately 1 g of chilled obex tissue was homogenized on ice in 9 mL of chilled extraction buffer (0.01 M PBS, 1% Nonidet P40, 10% w/v complete EDTA-free protease inhibitor cocktail tablets; Roche Diagnostics, Indianapolis, IN) with a Polytron (Brinkmann Instruments, Westbury, NY) homogenizer in a 50 mL polycarbonate tube. Homogenates were immediately centrifuged at 5,000 x g for 20 minutes at 4ºC. The clarified supernatant was analyzed for total protein 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 -80ºC until analysis.

All electrophoresis and Western Blot (WB) supplies were purchased from Invitrogen Corp. (Carlsbad, CA) unless otherwise stated. Polyacrylamide Gel Electrophoresis (PAGE) was performed using pre-cast NuPAGE Novex 10%

Bis-Tris gels and the Novex X-Cell Surelock Mini-Cell electrophoresis system. Magic Mark XP Western Protein Standard molecular weight marker was used for molecular weight (MW) estimation. To serve as positive and negative controls, recombinant PrP^c ($rPrP^c$) ab753 (Abcam Inc., Cambridge, MA) and water, respectively, were treated exactly as samples. Proteins were separated on gels under denaturing conditions using MOPS (3-(*N*-morpholino) propane sulfonic acid) SDS (sodium dodecyl sulphate) running buffer (pH 7.3-7.7) at 200V constant for 50 minutes. After electrophoresis the gel was gently removed from the casing and the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane using the X-Cell II Blot Module and NuPAGE Transfer Buffer with 10% methanol and 0.1% antioxidant at a constant 30V for 60 minutes. The WB was visualized using the Western Breeze Chemiluminescent Kit. All washes were done at room temperature using a shaker platform. Membranes were blocked and then exposed to 1:10,000 diluted anti-PrP^c mAb 6H4 (Prionics AG, Switzerland) for 1 h. After rinsing with antibody wash three times the membrane was incubated with the secondary antibody, anti-mouse conjugated to alkaline phosphatase, for 30 minutes. The membrane was thoroughly washed and then exposed to 2.5 mL of chemiluminescent substrate (CDP-Star) for 5 minutes. To capture the WB image, Kodak X-OMAT LS film (Eastman Kodak Co., Rochester, NY) was exposed to the membrane for 4 minutes in a dark room and was subsequently

developed using an auto-developer (Kodak X-OMAT Clinic 1 Processor, Eastman Kodak Co., Rochester, NY).

Membranes were then stripped using Restore Western Blot Stripping Buffer (Pierce Biotechnology, Inc., Rockford, IL) and probed for β-actin using anti-β-actin mAb AC-74 (Sigma-Aldrich, Inc., St. Louis, MO). Membranes were visualized using chemiluminescence as previously described. Beta-actin was used as an internal loading control to normalize all lanes within a gel.

The WB images were analyzed using Image Quant TL (Amersham Biosciences, Piscataway, NJ). Analysis included band identification, MW estimation based on standardized molecular weight markers, and relative optical densitometry for each glycoform of PrP^c. All WB analysis was based on two to three WB replicates.

To determine the effects of treatment on prion proteinase degradability, samples were first exposed to proteinase K (PK; Bio-Rad Laboratories, Inc., Hercules, CA) as described by Thackray et al. (2002) prior to PAGE and WB. Briefly, 10% brain tissue homogenates were digested with 250 ug PK/mL for one h at 37ºC. Both unexposed and PK exposed samples for each animal were run parallel to one anther on the same gel. The PK protocol described by Brown et al. (2000), using 0, 2, 10, and 25 ug PK/mL of 10% brain 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 and reducing agent and heating to

70ºC. Proteinase degradability was determined by comparing the WB elution profiles of PrP^c 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 enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical Co., Ann Arbor, MI) was used to quantitate PrP^c. Brain tissue homogenates (100 uL) were incubated in duplicate wells on a 96-well plate pre-coated with anti-PrP^c mouse monoclonal antibody for 2 h at room temperature. A standard curve was constructed using known quantities of $rPrP^c$ ab753. The secondary antibody, conjugated to acetylcholinesterase, was added and incubated for 2 h followed by the addition of substrate (Ellman's reagent). Color formation was allowed to develop for 30 minutes in darkness at room temperature. Absorbances were read at 405 nm using a plate reader (Synergy HT, Bio-Tek Instruments, Inc., Winooski, VT). Ellman's reagent was used as the blank and absorbances were corrected accordingly.

Total superoxide dismutase (SOD) activity of the brain tissue homogenates was measured using a SOD kit (Cayman Chemical Co., Ann Arbor, MI). Purified SOD was used to construct a standard curve for sample SOD activity quantification. Ten microliters of protein equilibrated brain tissue homogenate was added to duplicate wells in addition to 200 uL of the radical detector (tetrazolium salt). Addition of 20 uL of xanthine oxidase to each well and incubation for 20 minutes allowed for the formation of superoxide radicals

and subsequent color formation. Absorbances were read at 450 nm using a plate reader (Synergy HT, Bio-Tek Instruments, Inc., Winooski, VT). One unit (U) of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. Assays were performed in duplicate and SOD activities expressed as U/mg protein.

In addition to total SOD activity, Cu/Zn SOD and Mn SOD activities of the brain tissue homogenates were determined using the same assay. Potassium cyanide (KCN; 2 mM) was used to inhibit Cu/Zn SOD in the brain tissue homogenates, which subsequently allowed for the detection of Mn SOD activity. Copper/Zn SOD activity was then calculated by the subtraction of Mn SOD activity from total SOD activity (Brown and Besinger, 1998).

Brain tissue homogenates were also assayed for their overall antioxidant capacity (Antioxidant Assay Kit, Cayman Chemical Co., Ann Arbor, MI). The assay relies on the ability of antioxidants in the sample to inhibit the oxidation of 2,2'-azino-di-(3-ethylbenzthiazoline sulphonate) by metmyoglobin. The suppression of the absorbance, measured at 750 nm, is proportional to the concentration of the antioxidants which is expressed relative to trolox, a water-soluble tocopherol analogue. The assay was performed in duplicate.

Lipid peroxidation was measured in brain tissue homogenates by assaying for lipid hydroperoxides (LPO) using a lipid hydroperoxide assay kit (Cayman Chemical Co., Ann Arbor, MI). Lipid hydroperoxides are detected by their ability to oxidize ferrous ions. The resulting ferric ions are subsequently

detected using thiocyanate as a chromagen, which are monitored by measuring the absorbance at 500 nm.

Prion proteins from all brain tissue homogenates were purified using immunoprecipitation similar to that described by Brown et al. (1999). The mAb 6H4 was coupled to protein G-agarose (Sigma-Aldrich, Inc., St. Louis, MO) and subsequently mixed with brain tissue homogenates overnight at 4ºC in microtube spin columns. The beads were extensively washed and proteins subsequently eluted from the beads with the addition of 50 mM glycine (pH 4.0) and neutralized with 100 mM Tris-HCl (pH 8.0). The protein concentrations of the purified PrP^c eluates were determined using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA) and the purity confirmed by elution on polyacrylamide gels. The SOD-like activity of immunopurified PrP^c was determined as described for brain tissue homogenates. Immunopurified PrP^c eluates were also analyzed for Cu and Mn concentrations using flameless atomic absorption spectrophotometry (GFA-6500, Shimadzu Scientific Instruments, Kyoto, Japan).

Statistical Analysis

All data was analyzed using Proc MIXED in SAS (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. Interactions that

were not significant were removed from the model. All performance data and post-mortem brain, liver and prion data were analyzed as a randomized complete design with fixed effects of treatment, sex, and treatment x sex. Animal served as the experimental unit. When the treatment x sex interaction was not significant 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 using two pre-planned single degree of freedom 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 two calves died (one each from treatments -Cu and -Cu+Mn) due to *Haemophilus somnus* infection. These animals and all their data were removed from the analysis.

Results

Thirty d prior to calving liver Cu tended ($P = 0.07$) to be decreased in cows on treatments -Cu (123.5 mg Cu/kg DM) and -Cu+Mn (105.8 mg Cu/kg DM) compared to controls (174.9 mg Cu/kg DM). However, cows receiving treatments -Cu and -Cu+Mn were not Cu-deficient prior to calving as planned. Providing supplemental Mo in the free choice mineral proved to be ineffective in inducing a Cu deficiency as rapidly as needed. Once the cows assigned to

treatments -Cu and -Cu+Mn began receiving Mo in a daily supplement they subsequently reached a Cu-deficient status between d 60 and d 180 postpartum. Day 180 liver Cu concentrations averaged 21.0 and 19.6 mg Cu/kg DM for the -Cu and -Cu+Mn treatments, respectively, compared to 214.8 mg Cu/kg DM for controls.

All calves appeared to be healthy at birth, however, 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 weights were not different across treatments with an overall average of 240.2 kg. When analyzed as repeated measures, treatment ($P < 0.001$), d ($P < 0.001$), and the interaction of treatment x d ($P < 0.001$) affected plasma Cu concentrations in the calves (Figure 1). Both treatments designed to induce Cu deficiency (-Cu and -Cu+Mn) resulted in lower plasma Cu concentrations ($P \leq 0.02$) on d 0, 180, 350, and 440. Plasma Cu was further depressed in animals receiving -Cu+Mn compared to those receiving -Cu on d 350 ($P = 0.10$) and d 440 ($P < 0.001$).

Liver Cu in calves was affected by treatment ($P < 0.001$), d ($P = 0.01$), and an interaction of treatment x d ($P < 0.001$) when analyzed as repeated measures. Treatments -Cu and -Cu+Mn resulted in lower liver Cu concentrations ($P < 0.001$) compared to control animals on d 180, 350, and 440 (Figure 2). Ending liver Cu concentrations, from post-harvest liver samples, were lower in treatments -Cu and -Cu+Mn relative to the control group ($P = 0.001$; Table 2). Both treatments designed to induce Cu deficiency (-Cu and -

Cu+Mn) resulted in liver Cu concentrations of less than 20 mg/kg DM and would therefore be considered Cu-deficient (Underwood, 1981).

Liver Mn was affected by treatment ($P < 0.001$), d ($P < 0.001$), and treatment x d ($P = 0.03$). Cattle receiving high levels of Mn (-Cu+Mn) had higher liver Mn concentrations ($P < 0.001$) on d 350 and 440 compared to cattle receiving treatment -Cu (Figure 3). Within the Cu-deficient treatments, ending liver Mn was higher ($P = 0.001$) in cattle receiving treatment -Cu+Mn (Table 2).

Brain (obex) Cu and Mn concentrations followed similar patterns as liver Cu and Mn. Obex Cu was decreased ($P = 0.001$) in the Cu-deficient treatments compared to control cattle (Table 2). Obex Mn was higher ($P = 0.001$) in cattle receiving treatment -Cu+Mn compared to treatment -Cu.

Densitometric analysis of WB indicated that PrP^c band relative optical densities were similar across all treatments (Figure 4). Brain prion protein concentrations, as determined by ELISA, were also similar among treatments (Figure 5). Thus, based on both ELISA quantified and WB relative optical densities, PrP^c concentrations were not affected by brain Cu and Mn concentrations.

The relative distribution of the three glycoforms and their estimated MW were similar across the three treatments (Table 3). Additionally, based on visual analysis there were no apparent differences in the banding patterns of PrP^c from all brain tissue homogenates (Figure 6).

All prion proteins were completely degraded as determined by WB analysis when exposed to 10 and 25 ug PK/mL 10% brain tissue homogenate (Figure 7). However, 2 ug PK/mL 10% brain tissue homogenate allowed for some PrP^c to remain intact and visible on the WB. This visible band was present in all samples; likely due to a very low and less than saturable PK enzyme concentration. The PK test indicates treatment did not affect PrP^c proteinase degradability and that animals were BSE negative.

Brain (obex) tissue total SOD activity tended to be lower in the -Cu treatment (P = 0.08) compared to the -Cu+Mn treatment (Table 4). Cu/Zn SOD activity tended (P = 0.06) to be lower in the Cu-deficient treatments compared to the control. Alternatively, Mn SOD activity tended (P = 0.08) to be increased in the Cu-deficient treatments compared to control animals. The overall antioxidant capacity of brain tissue homogenates was similar across all treatments indicating brain metal perturbations did not compromise the antioxidant defense systems (Figure 8). Lipid hydroperoxides, resulting from lipid peroxidation, were assayed in brain tissue homogenates but all values were below the detection limit (0.25 nmol hydroperoxide) of the assay (data not shown).

Immunopurified PrP^c had similar SOD-like activity across treatments (Table 4) and did not differ in Cu concentration (Figure 9). Manganese was detectable at low levels (<1 ug/g) in some immunopurified PrP^c eluates, but most samples had undetectable levels of Mn (data not shown).

Discussion

Our initial objective of inducing Cu deficiency in newborn calves was unsuccessful as the dams were not deficient prior to 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 prior to weaning it is not clear when liver Cu concentrations were depleted below 20 mg Cu/kg DM. Plasma Cu concentrations in treatments -Cu and -Cu+Mn were also lower at weaning relative to controls, but were above the 0.5 ug/mL threshold that is indicative of Cu deficiency (Underwood, 1981). However, it has previously been reported that plasma Cu values may indicate cattle are Cu adequate while liver Cu concentrations are indicative of Cu deficiency (Mullis et al., 2003; Ahola et al., 2005). Thus, it is difficult to precisely identify the 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 and all of the finishing phase. Growth performance and Cu status of these cattle are further described in a separate paper (Legleiter and Spears, 2006).

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 mg Mn/kg DM to 16 mg Mn/kg DM, is similar to that seen in cattle fed supplemental Mn ranging from 0 to 240 mg/kg DM (Legleiter et al., 2005). Conversely, liver Mn was not affected in mature cows receiving similar levels of Mn in a free choice mineral (Legleiter et al., 2006b; Legleiter et al., 2006c). 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 brain Cu and Mn profiles. Brain Cu was decreased 60% and 72% for treatments -Cu and -Cu+Mn, respectively. Additionally, exposure to 500 mg Mn/kg DM in treatment -Cu+Mn resulted in a 31% increase in brain Mn compared to cattle receiving 20 mg Mn/kg DM (-Cu). These induced perturbations in both brain Cu and Mn are more pronounced than in previous studies using mature cows (Legleiter et al., 2006b; Legleiter et al., 2006c); thus, as expected young calves appear to be more susceptible to changes in brain metal concentrations than mature cows.

The perturbed brain Cu and Mn concentrations tended to alter the SOD activities of the brain. Similar to that reported by Legleiter et al. (2006b), Mn SOD activity in Cu-deficient animals tended to be increased compared to controls. However, unlike either study using mature cows (Legleiter et al.,

2006b; Legleiter et al., 2006c), 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, it appears that the brain tissue SOD activities were affected to a greater extent in these calves than in the mature cows previously described (Legleiter et al., 2006b; Legleiter et al., 2006c). This is likely a reflection of the more dramatically affected brain Cu and Mn concentrations in the younger calves compared to the mature cows. However, realizing no changes in the total antioxidant capacity of the brain tissue coupled with the lack of detectable lipid hydroperoxides indicates the altered brain Cu and Mn concentrations and subsequent effects on the SOD activities did not increase oxidative stress.

As seen previously (Legleiter et al., 2006b; Legleiter et al., 2006c), a decrease in brain Cu coupled with an increase in Mn had minimal effects on the biochemical properties of PrP^c. Most notably, there were no detectable changes in immunopurified PrP^c Cu and Mn concentrations, SOD-like activity, or PK degradability. This is in contrast to other research (Brown et al., 2000; Wong et al, 2001b; Deloncle et al., 2006) upon which this current hypothesis was built. Thus, the lack of detectable changes in PrP^c fails to support the hypothesis that a decrease in brain Cu with a concomitant increase in Mn results in a Mn for Cu substitution on PrP^c that alters the biochemical properties

of the prion proteins. This is in agreement with other studies we have conducted (Legleiter et al., 2006a; Legleiter et al., 2006b; Legleiter et al., 2006c) as well as other researchers who have questioned the importance of PrP^c in Cu metabolism and antioxidant defense systems (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 PrP^c *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., 2006b), inhalation of Mn may produce significantly higher brain Mn concentrations. Further, the PrP^c-bound Cu *in vivo* may not be readily replaced by Mn ions as has been reported to occur *in vitro.* Thus, the relationship between 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 in the biochemical changes to prion proteins that are relevant to TSE, particularly sporadic TSE. To our knowledge this is the first study that evaluated the relevancy of previous findings regarding Cu, Mn and prions, by exposing young calves to low Cu diets or low Cu diets 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 possibly more susceptible to perturbations in brain Cu and Mn and changes in prion biology, particularly since Cu is critical for brain development (Prohaska and Bailey, 1993; Prohaska, 2000).

In conclusion, an imbalance in dietary Cu and Mn, particularly high levels of Mn in Cu-deficient calves, can dramatically alter brain Cu and Mn concentrations, but does not change the biochemical properties of prion proteins. This study in conjunction with others recently conducted (Legleiter et al., 2006a; Legleiter et al., 2006b; Legleiter et al., 2006c) collectively questions the theory that dietary Cu and Mn are involved in the pathogenesis of TSE by altering PrP^c biology.

Implications

Exposing young calves to low copper diets results in decreased brain copper and exposure to low copper diets coupled with high dietary manganese increases brain manganese. However, these changes in brain Cu and Mn do not affect prion protein biology. Most importantly, the imbalances in brain Cu and Mn in this study did not allow for the formation of proteinase resistant prion proteins or any other biochemical properties indicative of infective prions. Thus, if BSE can occur sporadically, a dietary imbalance in Cu and Mn does not appear to be a causative factor.

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Table 1. Composition of the growing and finishing diets

	Growing	Finishing
	% DM	
Corn silage	86.88	---
Corn	---	83.83
Soybean meal	9.00	7.00
Cottonseed hulls	---	5.00
Urea	1.00	0.75
Calcium sulfate	0.80	0.80
Calcium carbonate	0.10	0.40
Salt	0.20	0.20
Vitamin premix[a]	0.01	0.01
Trace mineral premix[b]	0.01	0.01
Monensin[c]	---	+
Treatment supplement[d]	2.00	2.00

[a]Provided per kilogram of premix: 6,600,000 IU of vitamin A; 1,520,000 IU of vitamin D; and 6,600 IU of vitamin E.

[b]Provided per kilogram of premix: 288 g of Zn as $ZnSO_4$; 4.8 g of I as $Ca(IO_3)_2(H_2O)$; 1.9 g of Se as Na_2SeO_3; and 1.0 g of Co as $CoCO_3$.

[c]Provided 33 mg of monensin/kg DM.

[d]A ground corn supplement provided the following treatments: control (10 mg Cu/kg DM, 20 mg Mn/kg DM); -Cu (20 mg Mn/kg DM, 5 mg Mo/kg DM); -Cu+Mn (500 mg Mn/kg DM, 5 mg Mo/kg DM).

Table 2. Effect of dietary Cu and Mn levels on liver and brain Cu and Mn concentrations[a]

	Treatments				Contrasts[b]	
	control	-Cu	-Cu+Mn	SEM	control vs. -Cu & -Cu+Mn	-Cu vs. -Cu+Mn
					-- P values --	
Copper, mg/kg DM						
Liver	161.5	5.9	4.2	9.9	0.001	0.91
Obex	11.6	4.6	3.2	1.1	0.001	0.37
Manganese, mg/kg DM						
Liver	12.7	12.2	17.1	0.8	0.06	0.001
Obex	1.7	1.6	2.1	0.1	0.09	0.001

[a]Samples collected following euthanasia after 360 d on study.

[b]Pre-planned single degree of freedom contrast used to separate the Least Squares Means.

Table 3. Effect of dietary Cu and Mn levels on prion protein molecular weights and relative glycoform distributions[a,b]

	Treatments				Contrasts[c]	
	control	-Cu	-Cu+Mn	SEM	control vs. -Cu & -Cu+Mn	-Cu vs. -Cu+Mn
Molecular weight, kD[d]					*-- P values --*	
Diglycosylated	35.9	35.6	35.8	0.2	0.43	0.32
Monoglycosylated	32.3	32.2	32.4	0.1	0.95	0.34
Unglycosylated	27.1	27.1	27.0	0.1	0.87	0.88
Glycoform distribution, %[e]						
Diglycosylated	20.2	22.6	20.1	1.1	0.43	0.14
Monoglycosylated	23.6	24.0	22.1	1.4	0.73	0.36
Unglycosylated	56.1	53.4	57.9	2.2	0.85	0.19

[a]PrP^c has two glycosylation sites allowing for the presence of three glycoforms.

[b]Reported means from the analysis of two to three Western blots.

[c]Pre-planned single degree of freedom contrast used to separate the Least Squares Means.

[d]Estimated based on gel migrations relative to a standardized molecular weight marker using gel analysis software (ImageQuant TL, Amersham Bioscience).

[e]Estimated based on the relative optical density of each band relative to all immunoreactive PrP^c from that sample.

Table 4. Effect of dietary Cu and Mn levels on brain tissue homogenate and immunopurified prion protein superoxide dismutase (SOD) activities[a]

	Treatments				Contrasts[b]	
	control	-Cu	-Cu+Mn	SEM	control vs. -Cu & -Cu+Mn	-Cu vs. -Cu+Mn
Brain (obex)					-- *P values* --	
Total SOD, U[c]/mg[d]	71.2	63.0	72.4	3.6	0.43	0.08
Cu/Zn SOD, U/mg	56.2	45.3	49.6	3.7	0.06	0.42
Mn SOD, U/mg	14.9	17.8	22.9	2.4	0.08	0.16
Prion protein						
SOD, U/mg[e]	63.7	76.2	77.5	8.5	0.16	0.91

[a]All assays performed in duplicate or triplicate.

[b]Pre-planned single degree of freedom contrast used to separate the Least Squares Means.

[c]One unit (U) is equal to the activity required for 50% dismutation of the superoxide radicals.

[d]Expressed as U/mg of protein in the 10% brain tissue homogenate.

[e]Expressed as U/mg of protein in the immunopurified PrP^c eluate.

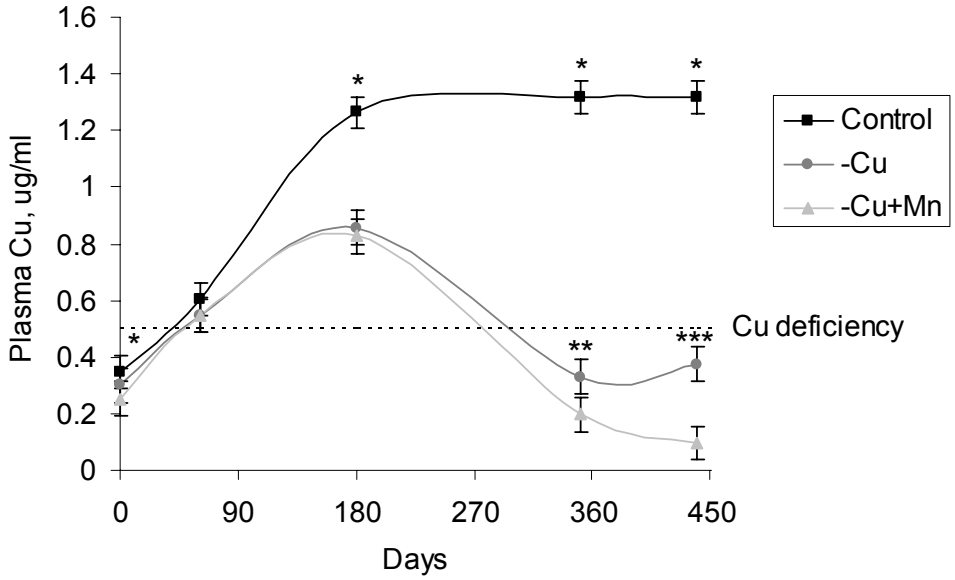

Figure 1. Average plasma Cu levels for each treatment from birth (d 0) to d 440. Calves were weaned (approximately 180 days of age) and started receiving the growing diet on d 210. Analyzing the data as repeated measures using Proc MIXED in SAS revealed main effects of treatment ($P < 0.001$), d ($P < 0.001$), and an interaction of treatment x d ($P < 0.001$). The interaction was further defined by analyzing the effect of treatment for each d using Proc MIXED in SAS and single-degree-of freedom contrasts to separate the means. Both treatments designed to induce Cu deficiency (-Cu and -Cu+Mn) resulted in lower plasma Cu concentrations (*Control vs. -Cu and -Cu+Mn, $P \leq 0.02$) compared to control animals on d 0, 180, 350, and 440. Plasma Cu was further depressed in animals receiving -Cu+Mn compared to those receiving -Cu on d 350 (**$P = 0.10$) and d 440 (***$P < 0.001$).

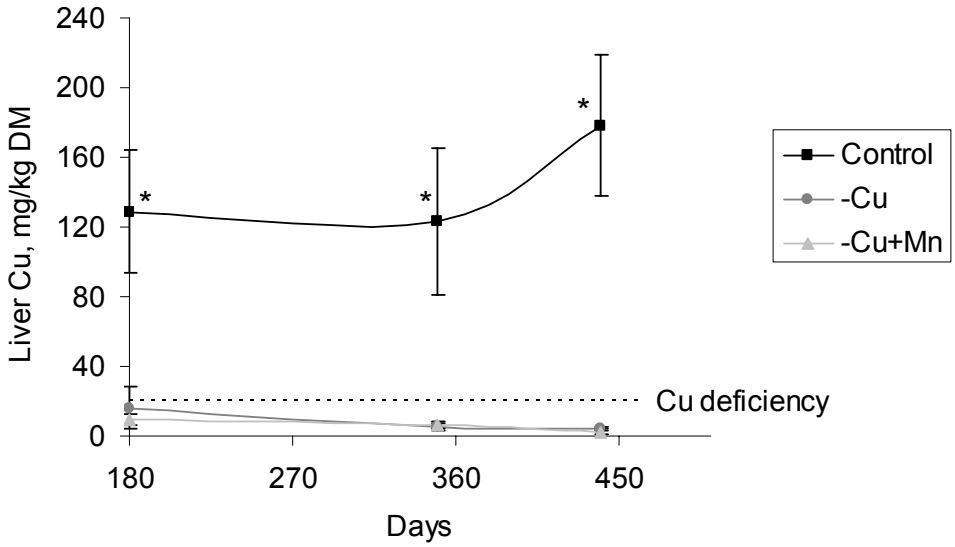

Figure 2. Average liver Cu stores for each treatment from d 180 to 470. Analyzing the data as repeated measures using Proc MIXED in SAS revealed main effects of treatment ($P < 0.001$), d ($P = 0.01$), and an interaction of treatment x d ($P < 0.001$). The interaction was further defined by analyzing the effect of treatment for each d using Proc MIXED in SAS and single-degree-of freedom contrasts to separate the means. Both treatments designed to induce Cu deficiency (-Cu and -Cu+Mn) resulted in lower liver Cu concentrations (*Control vs. -Cu and -Cu+Mn, $P < 0.001$) compared to control animals on d 180, 350, and 440.

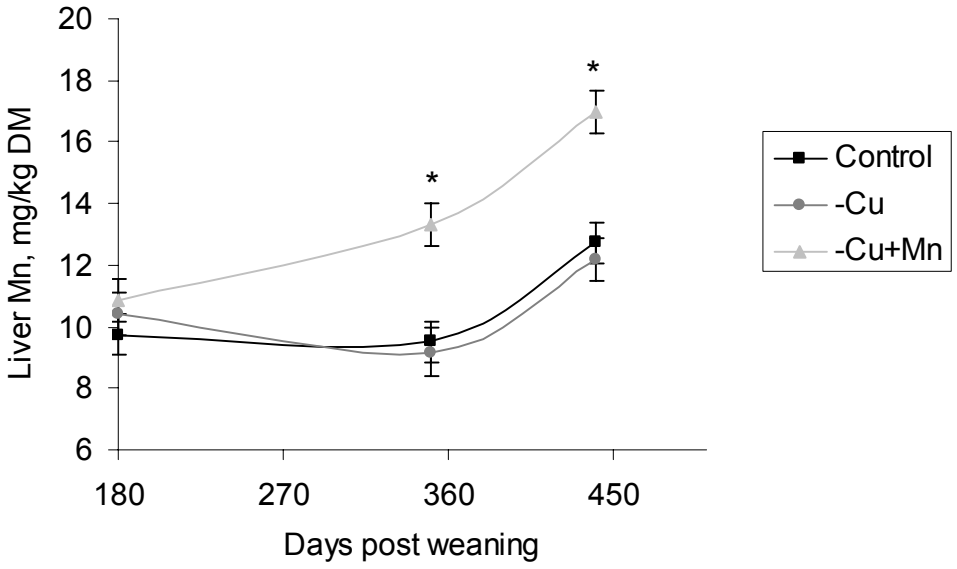

Figure 3. Average liver Mn concentrations for each treatment from d 180 to 470. Analyzing the data as repeated measures using Proc MIXED in SAS revealed main effects of treatment ($P < 0.001$), d ($P < 0.001$), and an interaction of treatment x d ($P = 0.03$). The interaction was further defined by analyzing the effect of treatment for each d using Proc MIXED in SAS and single-degree-of freedom contrasts to separate the means. Within the Cu-deficient treatments (-Cu and -Cu+Mn), cattle receiving high levels of Mn (-Cu+Mn) had higher liver Mn concentrations (*-Cu vs. -Cu+Mn, $P < 0.001$) compared to -Cu animals on d 350 and 440.

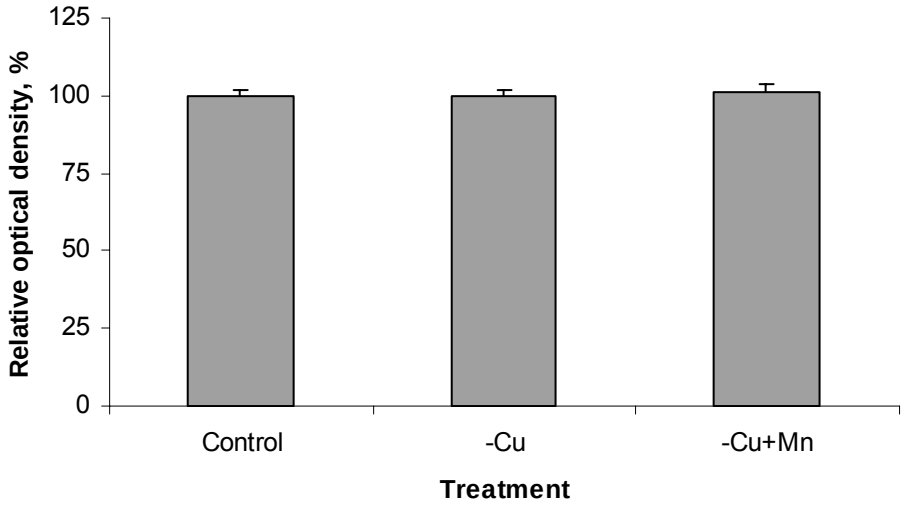

Figure 4. Effects of dietary Cu and Mn levels on relative concentrations of immunoreactive prion proteins. The relative optical densities of prion protein bands were determined by densitometric analysis of Western blots. Optical densities of -Cu and -Cu+Mn are expressed as a percent of the control.
control vs. -Cu and -Cu+Mn, $P = 0.84$
-Cu vs. -Cu+Mn, $P = 0.57$

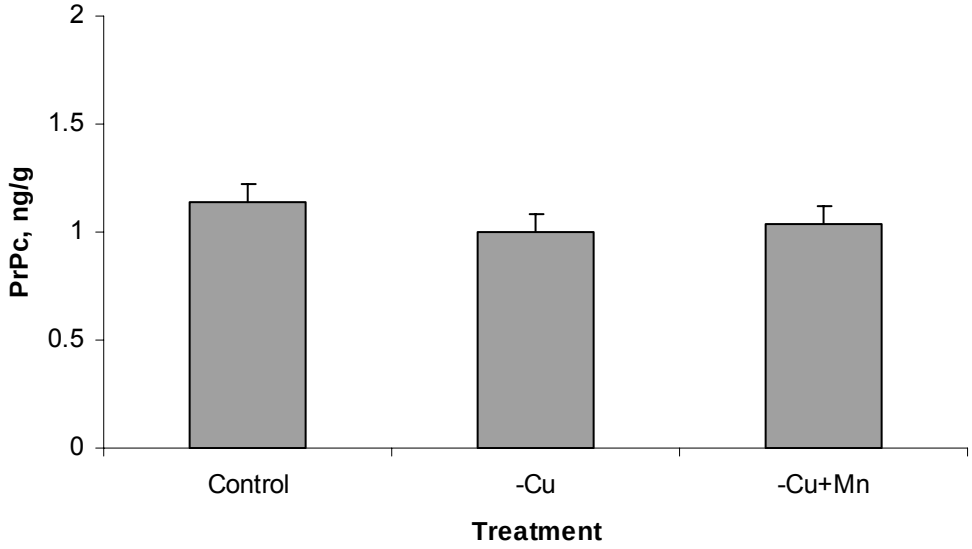

Figure 5. Effects of dietary Cu and Mn levels on brain prion protein concentrations. Brain (obex) prion protein (PrP^c) concentrations were determined by enzyme-linked immunsorbent assay.

control vs. -Cu and -Cu+Mn, $P = 0.24$

-Cu vs. -Cu+Mn, $P = 0.71$

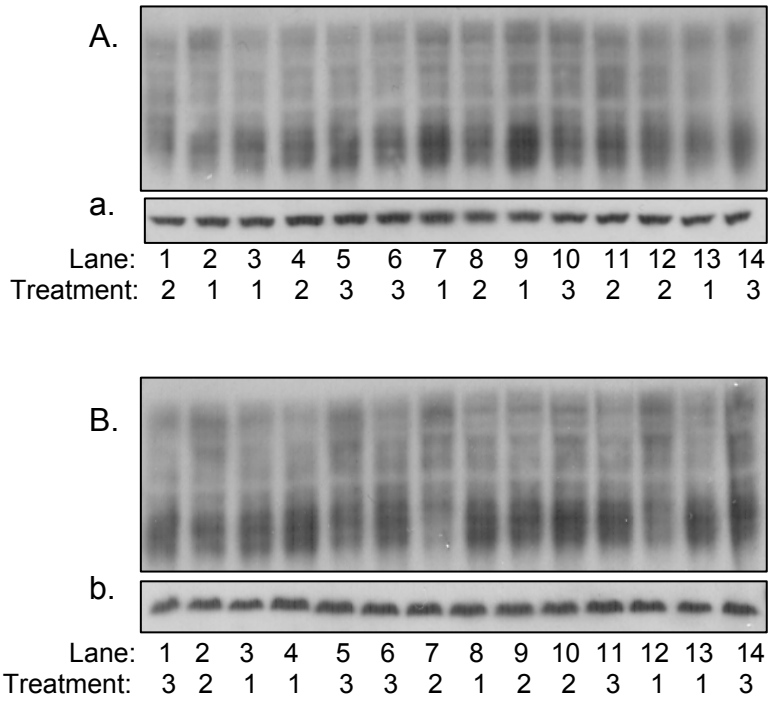

Figure 6. Effects of dietary Cu and Mn levels on prion protein elution profiles. The Western blots (WB) are of prion proteins (Panels A, B) from brain tissue homogenates from all 28 steers and heifers. Visual analysis of the WB shows similar prion protein banding patterns across treatments. Treatments: 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).

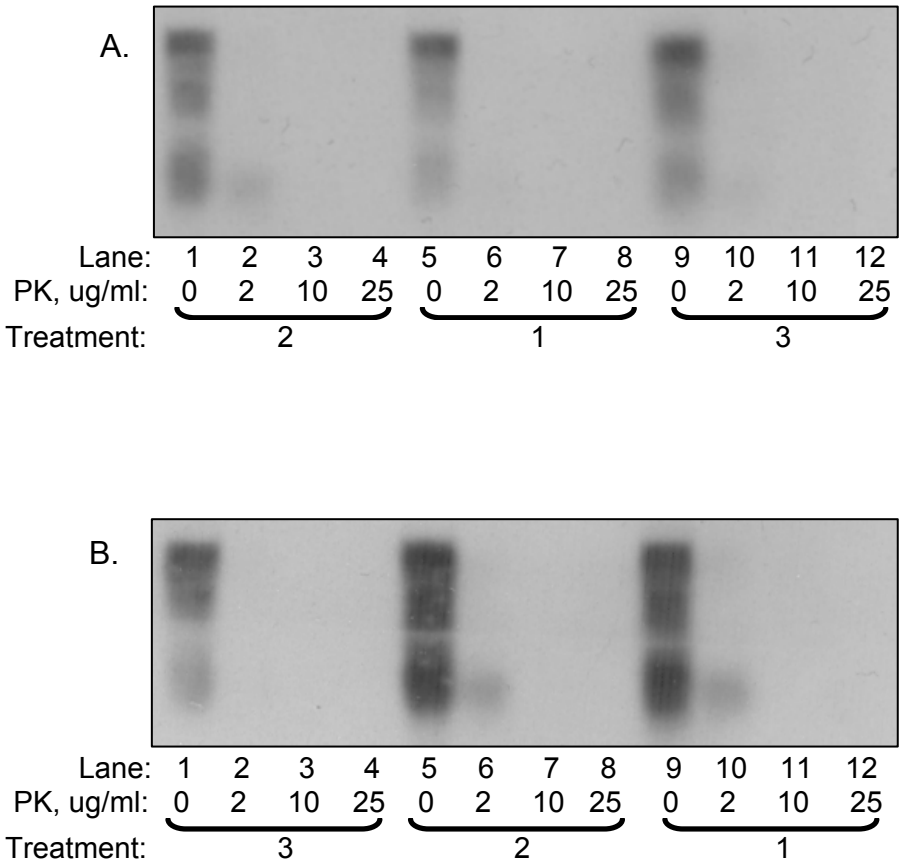

Figure 7. Effects of dietary Cu and Mn levels on prion protein proteinase degradability. The representative Western blots (WB) of prion proteins from brain tissue homogenates were exposed to 0, 2, 10, and 25 ug PK/mL 10% brain tissue homogenate for 30 minutes at 37°C. For both WB (A & B) shown here, as well as all other samples tested, 10 and 25 ug PK/mL 10% brain tissue homogenate completely degraded all prion proteins while 2 ug PK/mL 10% brain tissue homogenate degraded most but not all of the prion proteins in the sample. However, prion proteins from all animals across all treatments were degraded in a similar manner indicating treatment had no effect on PK degradability. Treatments: 1) Cu-adequate (control); 2) Cu-deficient (-Cu); 3) Cu-deficient plus high dietary Mn (-Cu+Mn).

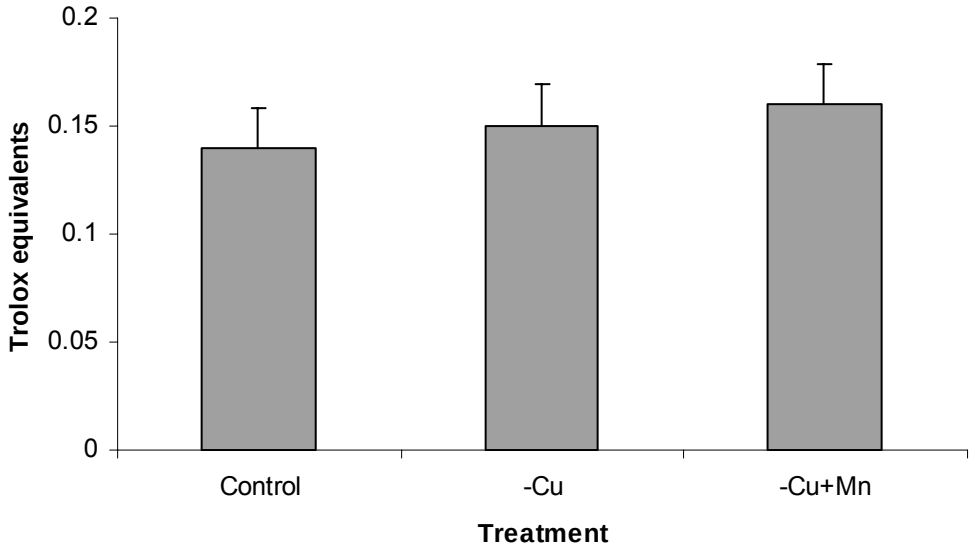

Figure 8. Effects of dietary Cu and Mn levels on brain tissue homogenate total antioxidant capacity. The antioxidant capacity of obex tissue homogenates was determined by the inhibition of ethylbenzthiazoline sulphonate oxidation by metmyoglobin. The antioxidant capacity is expressed as Trolox, a water-soluble tocopherol analogue, equivalents.
control vs. -Cu, $P = 0.39$
-Cu vs. -Cu+Mn, $P = 0.58$

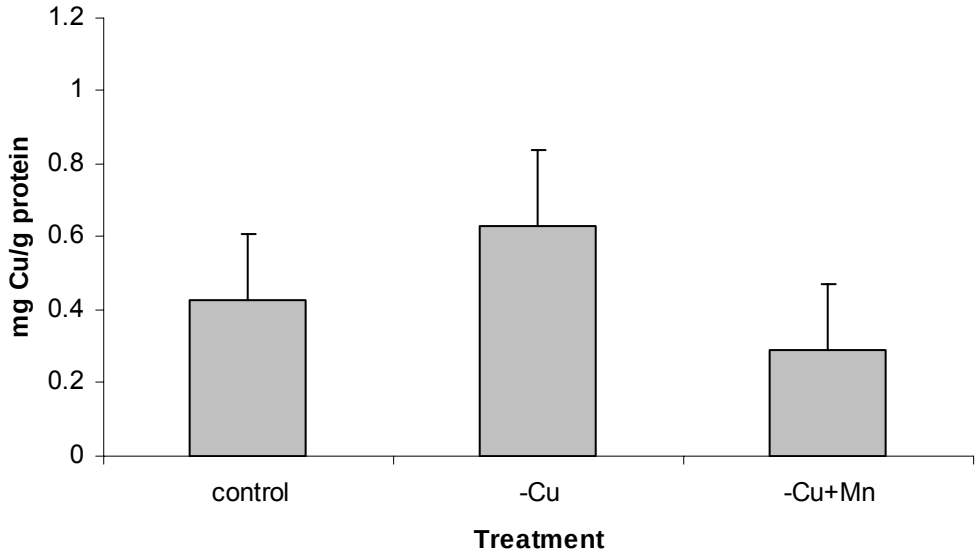

Figure 9. Effects of dietary Cu and Mn concentrations on immunopurified prion protein Cu content. Copper concentrations of immunopurified PrP^c eluates were determined by flameless atomic absorption spectrophotometry. Means are expressed as mg Cu/g protein.
control vs. -Cu and -Cu+Mn, $P = 0.87$
-Cu vs. -Cu+Mn, $P = 0.24$