Dietary magnesium and copper affect survival time and neuroinflammation in chronic wasting disease

Tracy A. Nichols\textsuperscript{a}, Terry R. Spraker\textsuperscript{b,c}, Thomas Gidlewski\textsuperscript{a}, Bruce Cummings\textsuperscript{b}, Dana Hill\textsuperscript{c}, Qingzhong Kong\textsuperscript{d}, Aru Balachandran\textsuperscript{e}, Kurt C. VerCauteren\textsuperscript{a}, and Mark D. Zabel\textsuperscript{c}

\textsuperscript{a}National Wildlife Research Center, US Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services, Fort Collins, CO, USA;
\textsuperscript{b}Colorado State University Diagnostic Laboratory, College of Veterinary Medicine, Colorado State University, Fort Collins, CO, USA;
\textsuperscript{c}Department of Microbiology, Immunology and Pathology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University Prion Research Center, Fort Collins, CO, USA;
\textsuperscript{d}Departments of Pathology and Neurology & National Center for Regenerative Medicine, Case Western Reserve University, Cleveland, OH, USA;
\textsuperscript{e}National and OIE Reference Laboratory for Scrapie and CWD, Canadian Food Inspection Agency, Ottawa, Ontario, Canada

\textbf{ABSTRACT.} Chronic wasting disease (CWD), the only known wildlife prion disease, affects deer, elk and moose. The disease is an ongoing and expanding problem in both wild and captive North American cervid populations and is difficult to control in part due to the extreme environmental persistence of prions, which can transmit disease years after initial contamination. The role of exogenous factors in CWD transmission and progression is largely unexplored. In an effort to
understand the influence of environmental and dietary constituents on CWD, we collected and analyzed water and soil samples from CWD-negative and positive captive cervid facilities, as well as from wild CWD-endozootic areas. Our analysis revealed that, when compared with CWD-positive sites, CWD-negative sites had a significantly higher concentration of magnesium, and a higher magnesium/copper (Mg/Cu) ratio in the water than that from CWD-positive sites. When cervidized transgenic mice were fed a custom diet devoid of Mg and Cu and drinking water with varied Mg/Cu ratios, we found that higher Mg/Cu ratio resulted in significantly longer survival times after intracerebral CWD inoculation. We also detected reduced levels of inflammatory cytokine gene expression in mice fed a modified diet with a higher Mg/Cu ratio compared to those on a standard rodent diet. These findings indicate a role for dietary Mg and Cu in CWD pathogenesis through modulating inflammation in the brain.

KEYWORDS. Cations, chronic wasting disease, copper, environment, iron, magnesium, manganese, metals, prion disease

INTRODUCTION

Chronic wasting disease (CWD), the only known wildlife prion disease, is a progressive fatal neurodegenerative disease that is transmitted in the absence of nucleic acids by infectious proteinaceous particles that resists protease digestion (PrPRes). Efficient transmission occurs directly from animal-to-animal and indirectly via the environment. Brain pathology includes PrP(CWD) plaques, neuroinflammation, and spongiform degeneration. Since its recognition in the 1960s, CWD has become a persistent problem in both wild and captive populations of North American cervids (deer, elk and moose), has spread to 22 states in the USA and 2 Canadian provinces and shows no sign of stopping. Once established, prevalence rates can be as high as 50% in deer, leading to an overall decrease in cervid populations. The presence of CWD has been catastrophic to the captive cervid industry, often requiring the culling of entire herds. The disease is difficult to control in part because prions are able to remain infectious in the environment and transmit disease years after contamination, providing an ongoing reservoir for indirect CWD transmission. There are currently no treatments or preventative available for CWD or any other prion disease.

Multiple studies have suggested that essential micronutrients, such as copper (Cu²⁺) and manganese (Mn²⁺), may have an influence on the conversion of PrP(C) to PrP(Res) with Cu²⁺ chelation resulting in delayed prion disease onset. Copper is a normal ligand for PrPC, and Mn²⁺ has been shown to actively compete for Cu²⁺ binding sites on PrPC. To understand the role of environmental and dietary constituents on CWD infection and pathogenesis, we collected and analyzed water and soil samples from CWD-positive and negative captive cervid facilities, as well as from CWD-endozootic wild areas. Our analysis revealed that water from CWD-negative sites had significantly higher magnesium [(Mg)/Cu, Mg/Mn, and Mg/iron (Fe)] ratios than water from CWD-positive sites, with the Mg/Cu ratio having the most significant relevance. The levels Mg in the water were higher on negative sites and Cu, Mn, and Fe were all lower on negative sites, although not statistically significant. To further assess the roles of Mg and Cu in CWD disease progression, a bioassay was conducted in which transgenic mice expressing elk PrP (Tg 12) were fed a custom diet devoid of Mg and Cu in combination with drinking water containing a spectrum of Mg/Cu ratios. Because the environmental Mg/Cu ratio had the highest significance, it was selected to be the focus of this study. Our results demonstrate that after intracerebral CWD inoculation, (1) Tg 12 mice on diets with the highest Mg/Cu ratio and lowest Cu concentration had significantly longer survival times than those on lower Mg/Cu ratios and higher levels of Cu; (2) Tg 12 mice on a high Cu diet produced significantly higher levels of several inflammatory cytokines when compared to mice on a low Cu diet; (3) Tg 12 mice on a diet with an optimal Mg/Cu ratio
showed a lower expression of inflammatory genes when compared with mice on a standard rodent diet. Our findings are consistent with the hypothesis that the observed increase in survival time in mice on diets with high Mg/Cu ratios is due to a decrease in neuroinflammation, a hallmark of prion diseases, since Mg has been shown to decrease neuroinflammation, while copper does the opposite.

RESULTS

Environmental Samples - ICP Soil Analysis

Soil levels of copper (Cu), manganese (Mn), magnesium (Mg), and Iron (Fe) were compared between CWD-positive and CWD-negative locations. No significant difference between sites was found in either the levels of each individual cation (Fig. 1) or the ratios of Mg/Cu, Mg/Mn, and Mg/Fe (Fig. 2).

Soil Composition Analysis

Analysis was done to determine if soil composition differed between sites, and no significant difference was detected in the relative amount of organic matter, quartz, silt, and clay (Table 1).

Environmental Samples - ICP Water Analysis

ICP-MS analysis of water from the study locations revealed no statistically significant differences in individual Mg, Cu, Mn and Fe concentrations. However, with the exception of Mg, the metal cation concentrations tended to be higher on CWD-positive sites (Fig. 3). When Mg/Cu, Mg/Mn and Mg/Fe ratios were calculated, CWD-negatives sites all had significantly higher ratios than CWD-positive sites (Fig. 4).

FIGURE 1. ICP-MS soil concentrations (ppb- parts per billion) of Mg (A), Cu (B), Fe (C) and Mn (D) from captive white-tailed deer and elk locations (n = 16). No significant difference in cation concentration was detected between CWD-positive and CWD-negative locations (Two-tailed Student's T-test, α = 0.05) Mg (p = 0.719), Cu (p = 0.580), Fe (p = 0.335) and Mn (p = 0.732).
Transgenic Mouse Bioassay - Intracerebral Inoculation

Survival

TG 12 mice IC inoculated with CWD were euthanized when they became symptomatic. Disease status was confirmed by Western blot analysis (data not shown). Mice fed the custom diet with the highest Mg/Cu drinking water ratio and the lowest concentration of Cu in drinking water, (Group D), lived significantly longer (average 136 days) than those on custom diets with more Cu (average 125–128 days) (Table 2).

To examine the role of the individual cations and Mg/Cu ratio in increased survival time, survival times were plotted against cation concentration or Mg/Cu ratio. Increased survival time was associated with increased Mg concentration and Mg/Cu ratio, and with decreased Cu concentration (Fig. 5). Although difficult to compare, the Mg/Cu ratio had the greatest slope (0.02600) as compared to Mg alone (0.00007) and Cu (−0.00023).

Mouse Brain ICP-MS

Mass spectrometry analysis showed that the concentration of Cu in the brains of
CWD-negative and CWD-positive mice from the same treatment group were not significantly different, demonstrating that CWD status did not affect the amount of detectable Cu in the brain (Fig. 6-A). In contrast, significantly higher Mg levels were observed in CWD-positive mice from group A (Fig. 6-B). The Mg/Cu ratio between CWD-negative and CWD-positive mice was significantly different only in group A, with the CWD-positive mice having a higher ratio (Fig. 6-C). The concentrations of Mg, Cu, and the ratio of Mg/Cu in the brains at terminal stage CWD-positive mice appeared to remain constant, regardless of survival time (Fig. 7).

**RT-PCR**

Mice fed the custom diet with the highest drinking water Cu/Mg ratio, group D, showed differential gene expression (>2-fold change) from mice fed the standard 5001 diet and water, group E. Both CWD-positive time points (60 DPI and terminal disease) from group D, as well as from CWD-negative animals, were compared to the CWD-negative and positive time points from group E. Comparison of negative controls between each group did not show much differentiation in gene expression, with group D having 2 genes increased, and 3 decreased, as compared to group E (Fig. 8A, Table 3a). Comparison between group E negative controls and group E, 60 DPI CWD-inoculated mice showed alterations in 14 genes, with 4 up-regulated, and 10 down-regulated (Fig. 8B, Table 3b) as compared to negative controls. The group E terminal mice had 9 genes upregulated and 6 downregulated, as compared to the group E negative controls (Fig. 8C, Table 3c). Although the numbers of altered genes is similar, there were only 4 genes in common between the 2 time points. Group D, 60 DPI

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**FIGURE 3.** ICP-MS water concentrations (ppb = parts per billion) of Mg (A), Cu (B), Mn (C) and Fe (D) from captive white-tailed deer and elk locations. No significant difference in cation concentration was detected between CWD-positive and CWD-negative locations (Two-tailed Student’s T-test, $\alpha = 0.05$). Mg ($p = 0.109$), Cu ($p = 0.227$), Mn ($p = 0.323$), and Fe ($p = 0.271$).
CWD-inoculated mice showed alterations in 7 genes, with one up-regulated, and 6 down-regulated as compared to group E negative controls (Fig. 8D, Table 3d). In contrast, the group D terminal mouse comparisons had 13 altered genes, with 4 upregulated and 9 downregulated (Fig. 8E, Table 3e). The two group D time points had 2 common altered genes. Group D, 60 DPI mice only shared 4 genes in common with group E, 60 DPI. Terminal mice from groups D and E shared 7 genes in common (ccl-19, ccl-3, ccl-9, csf2, IL13, Tnfsf-11, Tnfsf-13). The most striking difference between time points is at 60 DPI, where group D had far fewer gene alterations than group E.

To determine if differential gene expression was associated with disease within group D, CWD-negative group D control mice were compared to both 60 DPI and terminal group D mice. This comparison revealed only 2 gene alterations at 60 DPI (Fig. 9A, Table 3f), but 13 alterations at terminal disease (Fig. 9B, Table 3g).

**Immunohistochemistry**

Glial fibrillary acidic protein (GFAP) staining in the hippocampus was compared between groups D and E. The hippocampus was selected for comparison, as it was the most affected brain region in CWD mouse studies conducted in this laboratory. Others have noted the presence of PrP^{CWD} staining in the hippocampus, however the pattern was different possibly due to differences in inoculum.\(^\text{25,26}\) None of the CWD-negative controls for groups D or E (data not shown) showed any staining for PrP^{CWD} amyloid plaques. Plaques were visible at DPI 60 in both groups (data not shown), with group D appearing to have more staining. At terminal disease plaques could be seen in both groups.
All groups showed GFAP expression in the hippocampus, however group E had more staining at DPI 60 (Fig. 10 B).

**Protein Expression**

We next investigated whether the increases in transcripts encoding chemokines and their receptors and inflammatory cytokines in mice fed high Cu diets translated into increased expression of cognate inflammatory cytokines. We simultaneously measured levels of 11 cytokines in brains from mice fed a high Cu diet (E) or low Cu diet (D). Of the inflammatory cytokines analyzed, we detected significant increases of IFN\(\gamma\) and IL-1\(\beta\) in brains of mice fed a high Cu diet and terminally ill with CWD over mock-infected controls, whereas mice fed a high Cu diet and terminally ill with CWD over mock-infected controls, whereas mice fed a low Cu diet exhibited no increase in IFN\(\gamma\) and a significant decrease of IL-1\(\beta\) at 60 DPI and terminal disease (Fig. 11).

We observed no significant difference in expression of TNF\(\alpha\) and IL-6. We detected no granulocyte-monocyte colony stimulating factor (GM-CSF) in any mice from any group. We also observed significant reduction in expression of T cell-activating cytokines IL-2, IL-12p70 and IL-18, as well as the anti-inflammatory Th2 cytokine IL-13, in mice fed low Cu diets compared to mice fed high Cu diets. We detected no IL-4 or IL-5 in any mice from any group.

**DISCUSSION**

The goal of this work was to determine if differences observed in Mg and Cu water concentrations on CWD-negative and CWD-positive sites could be translated into a transgenic mouse bioassay to assess their effects on CWD progression. Numerous studies have shown that metals such as manganese and copper alter prion protein stability, leading to altered levels in diseased animals.\(^{16,27-30}\) Copper is a common environmental divalent cation and can be found in varying concentrations in soil and water,\(^{31}\) is a normal ligand for PrP\(\text{C}\), and increased Cu levels show convincing evidence for being a factor in CWD propagation.\(^{19,32-35}\) However, little work has been done to
specifically assess the levels of cations in the environment and their impact on CWD.

**Environmental Sampling**

Our environmental analysis showed no difference between copper (Cu), manganese (Mn), magnesium (Mg), and iron (Fe) soil levels at CWD-positive and negative locations. However, Mg was significantly higher at CWD-negative sites in the water, and there was a trend toward decreased Cu, Mn and Fe levels at these sites, and increased Cu, Mn and Fe on CWD-positive sites.

Water sources were compared to determine if the origin of the water, well versus surface water, had an impact on the cation levels since metals can leach from pipes into the water. CWD-negative sites had a greater percentage of well water sources than CWD-positive sites, yet had lower levels of Cu indicating that the increased level of Cu, Fe and Mn seen at CWD-positive sites was not a result of leaching from copper supply pipes. A study by Davies and Brown suggests that increased Mn can help protect the prion protein from degradation and increase its infective potential.\(^{36}\) Although not statistically significant, an increase in Mn was seen in water from CWD-positive sites.

Although Cu was the cation selected for this study, it is totally within the realm of possibility that similar survival outcomes could be observed by interchanging Mn or Fe with the Cu. If so, it would suggest that redox reactions with divalent cations, in general, could have negative impacts on disease progression, survival, and neuroinflammation.

Several studies have implicated clays in increased environmental prion persistence and

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**FIGURE 5.** Survival times (DPI-days post inoculation) versus Mg, Cu, or Mg/Cu ratio concentrations in CWD+ mouse diet. (A) Mg concentrations low to high, (B) Cu concentrations, low to high (C) and Mg/Cu expressed as a ratio, low to high. The Mg/Cu ratio showed the most positive influence on survival time (slope: 0.02600), followed by the negative influence of Cu (slope: –0.00023), and a modest increase on survival time by Mg (slope: 0.0007).
even infectivity. Soil composition was compared between positive and negative sites. The clay fraction levels were similar between sites suggesting that increased clay composition was not responsible for the increased incidence of CWD at the positive locations. A recent study examining the particulars of clay composition revealed that the type of clay present can influence CWD persistence. However, that type of analysis was beyond the scope of this study, and would not explain the bioassay results where no clay exposure occurred.

**Transgenic Mouse Bioassay**

Mice fed the lowest concentration of Cu and highest concentration of Mg, ie the highest Mg/Cu ratio, lived significantly longer than the other treatment groups with custom diets and supplemented drinking water, as well as the standard rodent diet and water group. Mice were inoculated with an infectious dose one order of magnitude lower than what is often utilized in IC studies (0.1% vs. 1% infected brain homogenate). This was done to try to more closely represent a natural dose. However, we readily acknowledge that the very nature of IC inoculation precludes it from being a true model for natural infection. It appears that Cu concentration had the greatest impact on survival, as mice fed the lowest concentration lived the longest. Mice fed >6000 ppb had similar survival times indicating a threshold dose between 2000 and 6000 ppb. However, all animals in each group ultimately succumbed to disease. Our results support findings from a previous study using mouse scrapie which demonstrated a neuroprotective effect of dietary copper depletion, but an inability to prevent neuronal death. However, a
balance is essential, as significant copper depletion can also have detrimental effects such as spongiosis in the brain. Copper also has been shown to play a role in Alzheimer’s disease (AD), a close cousin of prion diseases. Increased Cu has been found in the brains of AD patients, and been shown to increase amyloid precursor protein (APP) expression and exacerbate both tau and amyloid β pathology, while copper chelation slowed Alzheimer’s disease progression. The presence of redox-active metals, particularly Cu, has been shown to induce oxidative stress in the brain. Unlike Cu, Mg is not a ligand for binding sites on PrP. However, it is an important component for over 300 enzymes such as those that polymerize RNA and DNA, phosphorylate molecules, and combat oxidative stress. The role of Mg in prion disease has largely been ignored as it does not directly bind to the prion protein like Cu or Mn. However, a study with Alzheimer’s disease patients demonstrated that individuals who took Mg supplements, had slower cognitive decline than those that didn’t. Magnesium ions can reduce inflammatory responses and neuronal death by antagonizing calcium channels thus preventing
the release of neurotoxic TNFα and IL-6, and reducing pro-apoptotic proteins.61-63
Both TNFα and IL-6 protein levels were reduced in mice fed lower Cu and higher Mg in this study. Mg levels, overall, are reduced in patients with AD54,64 and Mg supplementation has been suggested as a therapeutic agent59,65, 66 An increase in Mg alone was insufficient to rescue or prevent death from CWD in this study. Group E had very high levels of Mg (2,100,000 ppb), but still did not survive significantly longer than the other groups. However, Mg may have played somewhat of a role in increased survival as there was no significant difference in survival time between group C and groups A and B despite there being over a 6-fold increase in the copper concentration in A and B, but the same level of Mg. More work is warranted to evaluate the effect of magnesium on CWD and other prion diseases.

Mouse Brain ICP-MS

Analysis of the brains from 3 randomly selected mice from each of the 5 treatment groups and inoculation status did not reflect the amount of Cu and Mg ingested in drinking water. Mice consuming higher levels of Cu in their drinking water did not present with higher Cu brain levels than those receiving 19 times less, indicating tightly controlled homeostasis. CWD status did not affect the amount of Cu detected in the brain, however, mice with CWD did have increased Mg levels, with 2 groups having significant differences. Perhaps this reflects an attempt by the body to combat neuroinflammation, which is central to prion disease progression21,67 Studies have shown both increases and decreases in blood and brain cation levels of prion-positive vs. negative deer, elk and rodents.46,53, 54 In contrast to our findings, a study examining Mg and Cu levels in the brain of CWD-negative and positive elk found decreased brain Mg levels in CWD-positive animals and no difference in
TABLE 3. Gene alterations >-2-fold by comparison group. Table shows gene abbreviation and full name. CWD- = CWD-negative animals, CWD+ = CWD-positive animals

| Compared Groups | Gene                                      |
|-----------------|-------------------------------------------|
| a. Group E CWD- vs Group D CWD-                 |
| Cxcl-15         | Chemokine (c-x-c motif) ligand 15          |
| Ifng            | Interfuron gamma                          |
| IL-1β           | Interleukin 1 Beta                        |
| IL-16           | Interleukin 16                            |
| IL-17B          | Interleukin 17 B                          |
| b. Group E CWD- vs Group E CWD+ 60 DPI          |
| ccl-4           | Chemokine (C-C motif) ligand 4             |
| ccl-6           | Chemokine (C-C motif) ligand 6             |
| ccl-19          | Chemokine (C-C motif) ligand 19            |
| ccl-20          | Chemokine (C-C motif) ligand 20            |
| Ccr-2           | Chemokine (C-C motif) receptor 2           |
| Ccr-4           | Chemokine (C-C motif) receptor 4           |
| Cxcl-5          | Chemokine (c-x-c motif) ligand 5           |
| Tnfsf-13b       | Tumor necrosis factor (ligand) superfamily member 13b |
| Fasl            | Fas ligand (TNF superfamily member 6)      |
| Bmp-2           | Bone morphogen protein 2                  |
| Spp1            | Secreted phosphoprotein 1, OPN             |
| IL-2γr          | Interleukin 2 receptor gamma               |
| IL-13           | Interleukin 13                            |
| IL-16           | Interleukin 16                            |
| c. Group E CWD- vs Group E CWD+ terminal disease |
| ccl-3           | Chemokine (C-C motif) ligand 3             |
| ccl-9           | Chemokine (C-C motif) ligand 9             |
| ccl-19          | Chemokine (C-C motif) ligand 19            |
| Ccr-10          | Chemokine (C-C motif) receptor 10          |
| Cxcl-15         | Chemokine (c-x-c motif) ligand 15          |
| Tnfsf-11        | Tumor necrosis factor receptor superfamily member 11b- osteoprotegerin |
| Tnfsf-13        | Tumor necrosis factor (ligand) superfamily member 13 |
| Csf-2           | Colony stimulating factor 2 (granulocyte-macrophage) |
| OSM             | Oncostatin M                              |
| Ifng            | Interfuron gamma                          |
| TNF             | Tumor necrosis factor α                    |
| IL-2rb          | Interleukin 2 receptor gamma               |
| IL-11           | Interleukin 11                             |
| IL-13           | Interleukin 13                            |
| IL-16           | Interleukin 16                            |
| d. Group E CWD- vs Group D CWD+ 60 DPI          |
| ccl-12          | Chemokine (C-C motif) ligand 12            |
| ccl-19          | Chemokine (C-C motif) ligand 19            |
| Tnfsf-11        | Tumor necrosis factor receptor superfamily member 11b- osteoprotegerin |
| Spp1            | Secreted phosphoprotein 1, OPN             |
| IL-1β           | Interleukin 1 Beta                        |
| IL-16           | Interleukin 16                            |
| IL-27           | Interleukin 27                            |
| Gene            |                                           |
| e. Group E CWD- vs Group D CWD+ terminal disease |
| ccl-3           | Chemokine (C-C motif) ligand 3             |

(Continued on next page)
This is not terribly surprising, as elk and mice likely process and store minerals differently. Altered brain cation levels do not necessarily provide evidence of a causative effect, but could simply represent a storage or processing dysregulation in the animal.

FIGURE 9. RT-PCR differential gene expression comparing CWD-infected group D time points to the CWD-negative controls from group D. At 60 DPI, mice showed little differentiation from control animals (A). At terminal disease there was an increase in gene alterations with 13 exhibiting > 2-fold change (B). (20X magnification.)

TABLE 3. Gene alterations >2-fold by comparison group. Table shows gene abbreviation and full name. CWD- = CWD-negative animals, CWD+ = CWD-positive animals (Continued)

| Compared Groups | Gene                                      |
|-----------------|-------------------------------------------|
|                 | ccl-9 Chemokine (C-C motif) ligand 9      |
| f. Group D CWD- vs Group D CWD+ 60 DPI | ccl-1 Chemokine (C-C motif) ligand 1      |
|                 | ccl-19 Chemokine (C-C motif) ligand 19    |
|                 | Tnfsf-11 Tumor necrosis factor receptor superfamily member 11b- osteoprotegerin |
|                 | Tnfsf-13 Tumor necrosis factor (ligand) superfamily member 13 |
|                 | Fasl Fas ligand (TNF superfamily member 6) |
|                 | Bmp-2 Bone morphogenic protein 2          |
|                 | Csf-2 Colony stimulating factor 2 (granulocyte-macrophage) |
|                 | Nampt Nicotinamide phosphoribosyltransferase |
|                 | IL-5ra Interleukin 5 receptor alpha       |
|                 | IL-7 Interleukin 7                        |
|                 | IL-13 Interleukin 13                      |
|                 | IL-33 Interleukin 33                      |
| g. Group D CWD- vs Group D CWD+ terminal disease | Spp1 Secreted phosphoprotein 1, OPN |
|                 | ccl-2 Chemokine (C-C motif) ligand 2      |
|                 | ccl-3 Chemokine (C-C motif) ligand 3      |
|                 | ccl-6 Chemokine (C-C motif) ligand 6      |
|                 | ccl-9 Chemokine (C-C motif) ligand 9      |
|                 | ccl-12 Chemokine (C-C motif) ligand 12    |
|                 | Ccr-1 Chemokine (C-C motif) receptor 1    |
|                 | Ccr-6 Chemokine (C-C motif) receptor 6    |
|                 | Tnfsf-13b Tumor necrosis factor (ligand) superfamily member 13b |
|                 | OSM Oncostatin M                          |
|                 | TNF Tumor necrosis factor alpha           |
|                 | IL-5ra Interleukin 5 receptor alpha       |
|                 | IL-13 Interleukin 13                      |
|                 | IL-33 Interleukin 33                      |
To determine if a decrease in neuroinflammation might be responsible for an increase in survival times, we evaluated the expression of an array of inflammatory transcripts. We observed significant changes in the levels of transcripts in the brain associated with inflammation in mice fed a high Cu vs. low Cu diet. Even before onset of clinical prion disease, we detected increased expression of transcripts encoding chemokines and their receptors in high Cu diet mice over.
mock-infected controls, and no increases in chemotaxis transcripts in low Cu diet mice. At terminal stages of CWD, high Cu diet mice also increased expression of inflammatory transcripts for Oncostatin M, TNF\(\alpha\), and IFN\(\gamma\). We observed no such increase in low Cu diet mice.

**Protein Expression**

Increased inflammatory transcripts translated to increased expression of inflammatory proteins IFN\(\gamma\), IL-1\(\beta\) and IL-18, as well as T cell activating cytokines IL2, IL-12p70 and the anti-inflammatory TH2 cytokine IL-13 in mice fed a high Cu diet. Conversely, we observed decreased expression of these cytokines in brains of mice fed a low Cu diet. Taken together, these data support our hypothesis that lowering Cu levels in mouse diets decreases cerebral inflammation resulting in significantly prolonged survival in prion-infected mice.
Immunohistochemistry

Glial fibrillary acidic protein (GFAP), an intermediate filament protein, staining represents expression of the protein by astrocytes. The expression of GFAP is utilized as a marker for reactive gliosis due to disease, CNS injury, or neurodegeneration. Mice in group D showed less GFAP staining at 60 DPI when compared to group E at the same time point (Fig. 10 B). When taken into consideration with the neuroinflammatory gene expression data, the reduction may be a physical representation of reduced overall brain inflammation and injury. However, reduction of cellular GFAP expression has been shown to be reduced by intravenous administration of Mg in traumatic brain injury in rats and our data indicates higher Mg brain concentrations in CWD-infected mice. Although group E had a considerably higher concentration of Mg than group D, it also has a much higher concentration of Cu, which perhaps overwhelmed any anti-inflammatory effects that Mg might have conveyed.

Little research has been done to evaluate the effect of Mg on neurodegeneration considering its importance in biological functions and its potential to reduce inflammatory processes. An increased Mg/Cu ratio altered disease progression by delaying neuroinflammation, and as a consequence disease progression. The results of this preliminary study suggest a role for dietary constituents in the progression of CWD and the importance of feed composition in disease management. The next step in our research is to test the optimal Mg/Cu ratio in a cervid species for efficacy. It is our hope that these findings may lead toward the development of a cation-modified diet to prevent or reduce CWD susceptibility in captive facilities.

METHODS AND MATERIALS

Environmental Samples and Locations

Soil and water samples were collected from 10 captive white-tailed deer locations (5 CWD-negative, 5 CWD-positive), and water from 4 CWD-positive captive elk locations and 2 from CWD-positive wild sites, totaling 11 CWD-positive sites and 5 CWD-negative sites. All of the negative sites had 2 different water sources providing 10 different samples, and 4 of the positive sites had multiple water sources providing a total of 16 different samples. Water sources included surface sources (streams, ponds, and wallows), and well sources (waterers). The historical prevalence of CWD at the positive captive locations ranged between 5–25% and the cervid CWD incidence rates on the wild sites ranged between 10–30%. There was no history of CWD at negative sites.

Environmental Sample Analysis - Soil and Water by ICP-MS

Approximately 50 g of soil was taken from 10 captive white-tailed deer sites and 30 ml of water was collected from each source and placed in 50 ml conical tubes containing 3 drops of nitric acid (Sigma) for preservation. Upon receipt, soil and water samples were sent to the Colorado State University analytical chemistry laboratory in Pueblo, Colorado for inductively coupled plasma mass spectrometry (ICP-MS) metals analysis. Water samples were analyzed utilizing EPA method 200.871 and 6020.72 Soil samples were analyzed utilizing EPA method 305173 and 6020A.72

Soil Composition Analysis

To determine soil composition, samples were sent to the Colorado State University Soil Laboratory in Fort Collins, Colorado for analysis. Organic matter, (Method 29–3.5.2, Walkely-Black procedure),74 Quartz (Method 3–5.3),74 silt, and clay constituent percentages were identified (Method 15- hydrometer method).75

Transgenic Mouse Bioassay - Intracerebral Inoculation

All procedures involving animals were performed to minimize suffering and were approved by the Institutional Animal Care and...
Use Committee at the USDA National Wildlife Research Center in accordance with the USDA Animal Welfare Act Regulation. CFR, title 9, chapter 1, subchapter A, parts 1–4. The transgenic mouse line (TG12) utilized in this study expresses the elk prion protein with methionine/methionine at codon 132. Mice were generated as previously described, and generally develop disease-associated ataxia at about 120 d post inoculation (DPI) after intracerebral inoculation (IC) with 1% brain homogenate from CWD-affected cervids. For the IC survival portion of the study, 4 groups of mice were placed at weaning on a custom Teklad magnesium (Mg) and copper (Cu)-deficient diet (Harlan/Teklad) and de-ionized Mg and Cu-supplemented drinking water, and one group on Purina 5001 rodent diet (Purina) and normal drinking water containing negligible Mg and Cu levels (90 and 4 ppb respectively) (Tables 4, 5).

Mice were maintained on the diets for 30 d prior to IC inoculation with either CWD-positive brain homogenate from a terminal elk or CWD-negative transgenic mouse brain homogenate (NBH). Inoculum was made by diluting 10% (wt/vol) brain homogenate as previously described and diluted to 0.1% in sterile filtered phosphate buffered saline (PBS) containing 100 units/mL penicillin and 100 μg/mL streptomycin (Invitrogen) diluted in inoculum to 1X, then placed at room temperature for 20 min. The 0.1% brain homogenate was selected to more closely resemble a natural dose than the common 1%. Mice were anesthetized with isoflurane gas and inoculated IC with 30 μl of inoculum 3 mm deep through the coronal suture, 3–5 mm lateral of the sagittal suture. CWD-positive mice from groups A-C were euthanized at terminal disease when they presented with severe ataxia. Control mice from these groups were euthanized at 200 DPI. Mice from groups D and E were euthanized at 60 DPI, and at terminal disease when they presented with severe ataxia. Control mice from groups D and E were euthanized at 60 DPI and at 200 DPI. Brains were removed at euthanasia, and half frozen at −80°C and half fixed in 10% formalin. Disease presence was assessed in CWD-positive and negative mice by Western blot analysis (data not shown).

**Mouse Brain Cation Analysis by ICP-MS**

A 10% (wt/vol) brain homogenate (CWD-negative and CWD-positive) was prepared with 100 mg of formalin-fixed brain in de-ionized water, as described above, for 3, randomly selected mice from each test group. Sodium hydroxide (NaOH) (Sigma) was added to each sample to a final concentration of 1M NaOH solution to inactive any prions present. Samples were allowed to sit at room temperature for 10 min and then neutralized to pH 7 by adding nitric acid. Samples were then sent to the Colorado State University analytical chemistry laboratory in Pueblo, Colorado for ICP-MS analysis (method 6020A and 3052 72,77) to determine the Mg and Cu concentrations present.

**Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)**

Mouse brains from groups D and E DPI 60, and terminal disease (including those utilized in the IHC and protein assay portions), from groups D and E were prepared for RT-PCR inflammatory arrays (Table 6). The cost of the RT-PCR arrays prohibited the examination of all mice from these groups. The number of mice used in each group varied, as the quality of some of the RNA samples was poor and generated unusable RT-PCR data. Negative control groups from DPI 60 and terminal mice

| Constituent | Custom Diet-ppb | 5001 Diet-ppb |
|-------------|----------------|--------------|
| Protein     | 177,000,000    | 285,000,000  |
| Fat         | 52,000,000     | 135,000,000  |
| Potassium   | 9,520,000      | 11,800,000   |
| Magnesium   | 200            | 2,100,000    |
| Iron        | 210,000        | 270,000      |
| Zinc        | 57,800         | 79,000       |
| Manganese   | 122,000        | 70,000       |
| Copper      | 500            | 13,000       |
| Selenium    | 400            | 300          |
were combined for each group, and served as Group D and E controls respectively for gene expression analysis. Extraction of RNA for each sample was done by following the instructions in the RNeasy® Mini kit (Qiagen). Prior to extraction, 20 mg of brain, and 350 μl of Qiagen buffer RLT with betamercaptoethanol (BME) were added to 1.5 ml tubes with 2.5 mm glass beads, and homogenized with a Blue Bullet homogenizer (NextAdvance) for 2 min on setting 7. Synthesis of cDNA was done by utilizing the RT² First Strand kit (Qiagen) as per directions, from extracted RNA. Once cDNA was synthesized, it was frozen at −80°C until needed. Premade inflammatory RT-PCR 96-well arrays were purchased from SABiosciences (PAMM-011Z format D). Plates were loaded by with 25 μl per well of a 102 μl of cDNA and 1350 μl of RT² SYBR® Green qPCR master-mix (SABiosciences) mixture. Each plate contained genes for 84 mouse inflammatory cytokines and receptors that mediate the inflammatory response, as well as 5 housekeeping genes, and 7 genomic DNA, reverse transcription, and PCR control wells (see supplemental data section for complete list of genes). One plate per mouse was amplified on a Bio-Rad CFX96 RT-PCR instrument utilizing the following conditions: Hotstart at 95°C for 10 min, then 40 cycles at 95°C for 15 sec, then 60°C for 60 sec with ramp rate set to 1°C/sec. Completed RT-PCR data was exported from the Bio-Rad CFX96 and uploaded to the SABiosciences data analysis website (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php) for analysis.

**Immunohistochemistry (IHC)**

One brain hemisphere from each of 3 representative mice from the CWD-positive and negative from groups D and E were examined by IHC at 60 DPI and terminal disease. The three brain hemispheres were formalin-fixed and paraffin-embedded, then sectioned at 5 μm and placed on positively charged microscope slides. The slides were deparaffinized and rehydrated in deionized water.

To prepare for PrP<sup>CWD</sup> visualization, slides were pre-treated in 95% formic acid (Sigma-Aldrich, St. Louis, MO) for 5 minutes at room temperature, rinsed in deionized water and placed in 0.1 M Tris buffer, pH 7.3. Antigen retrieval was performed in DAKO Target Retrieval Solution (DAKO, Denmark) at 121°C for 20 minutes. Slides were stained with a Leica Bond Max automated immunohistochemistry stainer (Leica Biosystems, UK) using a combination of a Mouse-on-Mouse

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**TABLE 5. Magnesium (Mg) and Copper (Cu) levels and number of mice per treatment group with (+) and without (−) chronic wasting disease. ppb- parts per billion, Custom-Teklad Mg/Cu deficient diet and supplemented drinking water 5001-standard Purina rodent chow and regular drinking water.**

| Test Group   | [Mg] ppb | [Cu] ppb | Mg/Cu Ratio | Number of mice CWD + | Number of mice CWD − |
|--------------|----------|----------|-------------|----------------------|----------------------|
| A. Custom    | 1,000,000| 38,340   | 26          | 9                    | 9                    |
| B. Custom    | 350,000  | 38,340   | 9           | 8                    | 5                    |
| C. Custom    | 1,000,000| 6,000    | 167         | 12                   | 6                    |
| D. Custom    | 1,000,000| 2,000    | 500         | 9                    | 8                    |
| E. Custom    | 2,100,000| 13,000   | 162         | 9                    | 6                    |

**TABLE 6. Number of mice for each group utilized in the inflammatory RT-PCR array and protein expression assay. With chronic wasting disease (+) and without (−). DPI- days post inoculation.**

| Test Group and CWD Status | Number of mice |
|---------------------------|----------------|
| D. CWD- controls          | 6              |
| D. CWD + DPI 60           | 3              |
| D. CWD + Terminal disease | 2              |
| E. CWD- controls          | 4              |
| E. CWD + DPI 60           | 4              |
| E. CWD+ Terminal disease  | 4              |
Polymer IHC kit (Abcam, Cambridge, MA) and the Bond Polymer Refine Detection kit (Leica) and visualized by horseradish peroxidase-DAB chemistry at room temperature. The staining protocol is as follows with appropriate rinses between steps: hydrogen peroxide block (Abcam) 10 minutes, rodent block (Abcam) 30 minutes, mouse anti-prion primary antibody (F99/97.6.1, VMRD, Pullman, WA) 1:250 dilution for 30 minutes, protein block (Background Eraser, Biocare Medical, Concord, CA) 10 min., M.O.M. HRP Polymer secondary antibody (Abcam) 15 minutes, Mouse anti-PrP(CWD) was visualized as brownish granular staining.

**GFAP**

The same mouse brain samples were used as the PrP(CWD) IHC above. To stain for glial fibrillary acidic protein (GFAP), a marker for astroglisis, the formalin-fixed paraffin-embedded mouse brain tissue was sectioned at 5 μm and placed on positively charged microscope slides. Slides were heated at 60°C for 30 minutes and loaded onto a Leica Bond Max automated immunohistochemistry stainer (Leica Biosystems, UK). Slides were dewaxed and antigen retrieval with ER2 solution (Leica) at 95°C for 20 minutes. Anti-GFAP rabbit monoclonal primary antibody (clone SP78, Cell Marque, Rocklin CA) 1:100 dilution was incubated at room temperature for 30 minutes. Anti-rabbit secondary antibody (Leica) was incubated at room temperature for 25 minutes. The Bond Polymer Refine Red Detection kit using alkaline phosphatase-fast red visualization was run and the slides were counterstained with hematoxylin. Hippocampal regions of the brain were reviewed for changes in stain intensity as this is the most affected brain region in this transgenic line.

**Protein Expression Assay**

Mouse brains were homogenized as described above to generate a 10% homogenate with following change: protease inhibitor cocktail (Roche) was added to PMCA buffer 1, as per product instructions, in both steps utilizing PMCA buffer rather than using PMCA buffer 2 for the second part of the protocol. Brain homogenate was then stored at −80°C until used in the BioPlex assay.

A mouse Th1/Th2 Extended ProcartaPlex™ Immunoassay (eBioscience, ref#: EPX110-20820-901) was used to measure 11 cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-12p70, IL-13, IL-18, IFN-γ, GM-CSF and TNF-α). Brain homogenate samples were subjected to centrifugation (5000 rpm for 10min at 4°C) and supernatant was collected and diluted 1:10 for cytokine analysis. Preparation of samples, along with kit standards, detection antibody and streptavidin-PE, were carried out per the eBioscience magnetic immunoassay protocol. Standards, samples and a control were run in triplicate with CWD-negative and positive samples run on separate 96-well untreated polystyrene microplates (BD Falcon, 353910). Cytokine fluorescence intensity was detected using the BioPlex machine with Luminex Multiplexing Technology and analyzed by BioPlex Manager software version 5.0.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

No potential conflicts of interest were disclosed.

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