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Research Article

Copper Accumulation in Wisconsin Holsteins with Indications of Oxidative Liver Damage

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Abstract

An extensive review of Wisconsin Veterinary Diagnostic Laboratory(WVDL) accessions along with the results of slaughter house surveys indicate that copper is accumulating in the livers of Wisconsin Holsteins of all ages. Two hundred eleven WVDL accessions were reviewed based on breed(Holstein), age and results of inductively coupled plasma-mass spectroscopy(ICP-MS) liver element testing. The overall (all ages) mean liver copper(Cu) was 144ppm wet weight(ww). Animals > 2 years averaged 145ppm ww, those 1-24 months averaged 174ppm ww, those birth-30 days averaged 119ppm ww and fetuses averaged 95ppm ww. The average liver Cu for 45 fresh samples from cull dairy cows procured at a slaughter plant was 163ppm ww. This population may have included a few colored dairy cows (Jersey, Guernsey and Brown Swiss). Excess copper is thought to enter into reactions that result in the production of oxygen radicals, particularly the very toxic hydroxyl radical. These radicals damage the lipid moiety of cellular membranes, a form of oxidative stress. 4-hydroxynonenal (4-HNE) is one of many compounds that are generated by the reaction of oxygen radicals with the lipid moiety of cellular membranes. Immunohistochemical staining of livers with excess copper for 4-HNE is often positive, suggesting copper related oxidative damage. Copper is accumulating in the livers of Wisconsin Holsteins of all ages and may be causing oxidative liver damage.

ABBREVIATIONS

ppm ww: parts per million wet weight; **ICP-MS**: inductively coupled plasma-mass spectroscopy; **4-HNE**: 4-hydroxynonenal; **ROS**: reactive oxygen species; **H&E**: hematoxylin and eosin;

INTRODUCTION

This report documents the accumulation of copper in the livers of Wisconsin Holstein cattle of all ages and attempts to address the questions of, whether or not, affected livers incur oxidative stress or sustain damage evident by light microscopy. Oxidative stress is a complex, multifaceted process that could result in liver damage, interference with immune function or decreased productivity.

Copper is a transition metal that is essential for all life. Its redox properties bestow it with capabilities that are simultaneously essential and potentially damaging to the cell. Copper displays four oxidation states and no other element provides the redox properties embodied in copper. Its essentiality thus derives from its incorporation into a diverse variety of enzymatic and structural proteins that function in cellular respiration, free radical defense, cellular iron metabolism, connective tissue synthesis, pigmentation, blood clotting, peptide hormone production and neurotransmitter synthesis. It is potentially damaging because excess copper is toxic and, under normal conditions, free copper is virtually nonexistent within cells. When present, free copper has the very real potential, by virtue of its redox properties, to catalyze the formation of the highly toxic hydroxyl radical via the Fenton reaction [1]. Copper can also bind to and damage cellular proteins [2] and DNA [3]. It has been suggested that the cytotoxic effects of copper are attributable to nuclear damage with subsequent apoptosis and phagocytosis [4]. Apoptosis has been a consistent ultrastructural [5] and light microscopic [6] finding in affected livers. Because of this reactivity and the necessity to protect cells from free copper, an elaborate system of transporters and chaperones are present in both the intestine and liver (as well as certain other tissues) that govern absorption, transport, utilization and excretion. The necessity of these safeguards becomes readily apparent when, by virtue of genetic failure or being overwhelmed by excess copper, the system fails. Wilson disease is a devastating liver(among other organs) condition of young humans caused by mutations

of the ATP7B gene which encodes a copper transporting ATPase located on the biliary canalicular membrane. Affected people are unable to excrete excess copper resulting in hepatitis and, in many cases, eventual liver failure. As a model for Wilson disease, the Long-Evans cinnamon rat suffers a similar fate and, interestingly, many rats surviving the resulting hepatitis succumb to malignant liver cancer. Mutation of the, as yet incompletely understood, COMMD1 gene in Bedlington terriers causes hepatic copper accumulation with resulting hepatitis and it seems likely that other canine breeds are similarly affected by different mutations, though this is less clear [7]. By virtue of their inability to increase copper excretion in response to increased intakes, sheep are very susceptible to copper toxicity while pigs are so tolerant of copper that they have been fed, as a growth promotant, diets containing up to 250ppm copper(requirements are in the 10-15ppm range). Neonatal and milk fed animals appear to be more susceptible to copper toxicity than their adult counterparts, possibly due to higher efficiency of copper absorption and immature excretory pathways[4].

UPTAKE, TRANSPORT, UTILIZATION AND EXCRETION OF COPPER

Ingested copper exists in the intestine as the relatively stable, oxidized Cu^{++} which is reduced by enterocyte plasma membrane reductases to Cu^{+}. The highly specific copper transporter Ctr1, which is conserved from yeast to humans, transports Cu^{+} into the enterocyte[1] from which it is transported into the portal circulation by the transporter ATP7A. In the blood, copper is bound to small molecules such as his tidine and to serum macroglobulins and albumin for transport to the liver [7]. Upon arrival at the liver, copper is transported across the plasma membrane by the same Ctr1 whereupon the picture becomes a little fuzzy. Some or, perhaps, all the copper is initially sequestered by glutathione and metallothionein(also functions as a storage protein). Or some may be diverted directly to end use destinations or the excretory pathway. In any event, several chaperones come into play including:

1)CCS-which shuttles copper to the antioxidant enzyme Cu, Zn superoxide dismutase.

2)COX17-which provides copper to cytochrome C oxidase.

3)ATOX1-which delivers copper to ATPB7 located in the trans-Golgi compartment from whence it is incorporated into ceruloplasmin or excreted via the bile[7].

HEPATOTOXICITY OF COPPER

The organism has in place several safeguards to protect against excess copper. When exposed to excess copper, intestinal Ctr1 is rapidly endocytosed, presumably as a mechanism to limit uptake [1]. The liver attempts to limit copper accretion by dramatically increasing the production and copper saturation of metallothionein, thus enhancing storage and by massive release of ATPB7 from the trans-golgi network in an attempt to facilitate biliary excretion [1]. Toxicity can be expected when these mechanisms are disabled or overwhelmed. The following sequence of events has been proposed as the mechanism of copper toxicity. Copper accumulates in the cytosol causing high concentrations of highly copper saturated metallothionein which is taken up into lysosomes and incompletely degraded and polymerized. This forms an insoluble material containing reactive copper, stainable by rhodanine (metallothionein bound, cytosolic copper does not stain with rhodanine). This reactive copper, likely together with iron (also a highly redox active metal) initiates, via free radical production, lipid peroxidation of lysosomal membranes with subsequent release of hydrolytic enzymes and necrosis of the hepatocyte [8]. This is likely to release reactive copper and iron into the circulation precipitating the hemolytic crisis often observed or the methemoglobinemia sometimes observed. At this point, reactive copper would also accumulate in the kidneys resulting in tubular damage. The reactive copper and iron in the parenchyma along with the hypoxia created by intravascular hemolysis would then fuel massive liver failure.

OXIDATIVE STRESS

Oxidative stress is defined as an imbalance between the production of reactive oxygen species (ROS) and their removal by the antioxidant system, resulting in damage to cells and biomolecules along with interference to cellular signaling. Low levels of oxidative stress can also promote cellular proliferation [9]. Reactive oxygen species, in particular the superoxide radical, normally leak from the mitochondrial respiratory chain and pose an ongoing disposal challenge to the antioxidant system [10]. There are many other endogenous as well as exogenous sources of ROS. There is much to suggest that oxidative stress induced cellular damage is mediated by products of ROS induced peroxidative degradation of membrane polyunsaturated fatty acids. The lipid hydroperoxides produced subsequently decompose and generate an array of breakdown products that are capable of inflicting widespread cellular damage. Among these is the aldehydic molecule, 4-hydroxy-2-nonenal (4-HNE). This, now well characterized, molecule produces many adverse cellular effects, not the least of which is alteration of the level of cellular glutathione, the organism's primary redox regulatory molecule [11].

Thus, this report documents the hepatic accumulation of copper in Wisconsin Holsteins and attempts to link excess copper to oxidative liver damage as determined by immunohistochemical staining of 4-HNE.

MATERIALS AND METHODS

Two hundred eleven WVDL accessions were reviewed based on breed (Holstein), age and results of ICP-MS liver element analysis. When possible, hematoxylin and eosin (H&E) stained sections of livers were reviewed.

Forty five fresh livers from cull Holstein cows(may have included a low percentage of colored dairy breeds also) were obtained from a slaughter plant and analyzed for copper by ICP-MS. Samples were fixed in 10% formalin solution, cut into 5 micron thick sections and stained with H&E for histologic examination.

ICP analysis-

Liver samples were blended into homogenous mixtures prior to sampling. Approximately 0.5 grams of liver is weighed into

a microwave digestion vessel with 6 mL of approximately 40% nitric acid solution. Samples are then digested by microwave before loaded onto the inductively coupled plasma with mass spectroscopy (ICP-MS) instrument. After dilutions, the samples are run on the ICP-MS and analyzed using a calibration range of 0.05 ppm to 100 ppm of copper. Check standards are run during the same run and must be between 80-120% recovery.

4-HNE immunohistochemistry- Tissues were fixed in a 10% buffered formalin, processed, and embedded with paraffin. Sections were cut at 5 µm and placed on Leica Apex adhesive slides. Sections were deparaffinized and rehydrated. In order to block endogenous peroxidase activity the sections were incubated with Peroxidazed (Biocare Medical) for 5 minutes and washed with Tris-buffered saline with Tween 20 (TBST). Non-specific binding was reduced with Background Punisher (Biocare Medical) for 7 minutes. The sections were then incubated for 60 minutes with the primary antibody (4-HNE, 1:1000) at room temperature. All sections were washed again with TBST and then incubated with MACH2 goat anti-rabbit-HRP secondary antibody for 30 minutes. A final wash in TBST was followed by color development with Betazoid DAB (Biocare Medical) for 90 seconds. A 1:8 dilution of CAT Hematoxylin (Biocare Medical) was used to counterstain the sections for 1 minute. All slides were then dehydrated through graded alcohols, cleared in xylene and coverslipped.

RESULTS AND DISCUSSION

The 211 WVDL accessions were broken into 4 age-based groups. The mean liver copper of all animals (n=211) was 144 parts per million wet weight(ppm ww), median(med)=130 and standard deviation(SD)=86. (Adequate bovine liver copper is stated as 25-100ppm ww while the high-toxic range is 200-800ppm ww [12,13]) That of animals greater than 2 years of age(n=83) was 145ppm ww, med=140 and SD=78, that of animals from 1-24 months(n=55) was 174ppm ww, med=150 and SD=110, that of animals from birth-30 days(n=52) was 119ppm ww, med=110 and SD=45 and that of fetuses(n=21) was 95ppm ww, med=86 and SD=53.

The mean liver copper of the 45 cull cow livers was 163ppm ww, med=149 and SD=74. Histopathology- Figure 1 illustrates positive copper staining with rhodanine in liver of Holstein cow with liver copper of 420ppm ww. The staining is most evident around a central vein which is the typical copper accumulation pattern. Figure 2 illustrates positive immunohistochemical stain for 4-HNE in a pattern similar to that of copper.

The results of this study indicate that excessive copper is accumulating in the livers of Wisconsin Holsteins of all ages. The reason(s) for this is/are unclear but it is clear that baseline diets are often formulated to contain in excess of 20ppm copper on a dry basis. With this in mind, high producing cows with correspondingly high feed dry matter intakes will consume an amount of copper far in excess of their requirement and, since milk has a low copper content, this excess will not be offset by loss thus setting the stage for hepatic accumulation.

Many of the cull cow livers stained positive for 4-HNE in a pattern strikingly similar to the distribution of copper (compare the staining in figures 1&2), suggesting a connection. However, there was no apparent correlation between liver copper level

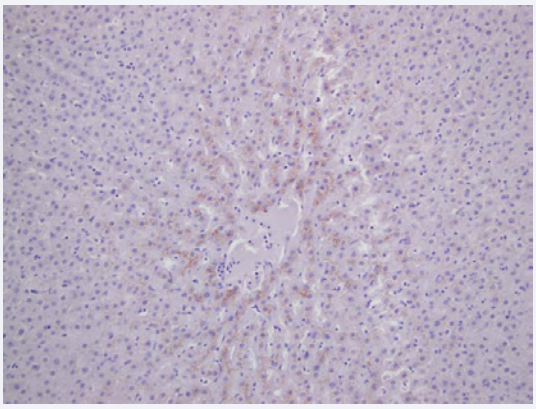

Figure 1 Liver(100X): Positive copper staining of hepatocytes around central vein. Rhodanine stain.

Figure 2 Liver(100X): Positive immunohistochemical stain for 4-HNE around central veins.

and the occurrence or intensity of 4-HNE positivity. This likely indicates that not all copper loaded livers are susceptible to the membrane lipid peroxidation that 4-HNE positivity represents, which is also suggested by the observation that histologic liver damage was usually minimal to absent even in livers with large excesses of copper. It may also be the case that additional or additive oxidative stressorscontribute to the 4-HNE positivity in livers that do not contain large excesses of copper. For instance, excess iron was evident in many cull cow livers and this element is known to also, under some conditions, be an oxidative stressor. Additionally, it is not known whether or not, copper induced oxidative stress always results in membrane lipid peroxidation and, thus, HNE positivity. This may depend on vitamin E status as this is the main antioxidant defense in lipid membranes. Or, more likely, the conditions predisposing to 4-HNE positivity, with or without excess copper, are unknown. This study fails to establish a connection between copper excess and oxidative stress, as represented by 4-HNE positivity. An additional indicator of oxidative stress appears necessary to prove a connection and hepatic glutathione would be a good candidate as its role as the bodies primary redox buffer makes it a sensitive indicator of oxidative stress. We were, regrettably, unable to assay it from liver.

CONCLUSION

Copper is accumulating in the livers of all ages of Wisconsin and, likely nationwide, Holsteins. The hypothesis that copper excess would cause oxidative damage as represented by 4-HNE positivity was not borne out but the striking resemblance of the patterns of copper staining and 4-HNE positivity in many livers with excess copper does suggest a connection. Histologic damage was, by and large, not evident in livers with excess copper. The fact that many livers were 4-HNE positive in the absence of excess copper suggests that oxidative damage is common and begs the question of what may be causing it in the absence of copper excess.

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