THE ASSOCIATION OF HEPATIC COPPER CONCENTRATION WITH HEPATOCYTE HEALTH AND OXIDATIVE STRESS IN DAIRY CATTLE

By

Jaimie Marie Strickland

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ABSTRACT

THE ASSOCIATION OF HEPATIC COPPER CONCENTRATION WITH HEPATOCYTE HEALTH AND OXIDATIVE STRESS IN DAIRY CATTLE

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Copper is sequestered in liver of all animals, and particularly so in ruminants, as an apparent reserve supply to provide for nutritional needs during times of insufficient dietary intake. Copper has poor dietary bioavailability in cattle and therefore copper is often supplemented in the diets of dairy cattle and over-supplementation can occur. Excessive hepatic copper accumulation in dairy cattle has been of growing concern in recent years. Despite this, the impact of elevated copper concentrations on bovine hepatocytes, short of fulminant toxicosis, is still not well understood. The overarching goal of this research is to provide veterinarians and nutritionists with a better interpretation of liver copper concentrations and their relevance to animal health, especially when they are above expected reference values but below fulminant toxic concentrations. The direct aim of this study is to determine the association between hepatic copper concentrations and biomarkers for subclinical hepatocellular damage in dairy cattle. Hepatocellular damage will be assessed by histological analysis, measurement of liver leakage enzymes in serum, and systemic and hepatic markers of oxidative stress. If such an association is found, it will suggest that super-nutritional, but sub-toxic hepatic copper concentrations may be a risk factor for disease in dairy cattle.

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KEY TO ABBREVIATIONS

CCO	Cytochrome c oxidase
SOD	Super oxide dismutase
GLDH	Glutamate dehydrogenase
GGT	γ-Glutamyltransferase
SDH	Sorbitol dehydrogenase
AST	Aspartate aminotransferase
BA	Bile acids
ICP-MS	Inductively coupled plasma mass spectrometry
AA	Atomic absorption
CCS	Copper chaperone for super oxide dismutase
Atox1	Antioxidant 1 copper chaperone
COX17	Cyclooxygenase
TGN	Trans-golgi network
MT	Metallothionein
CP	Ceruloplasmin
RONS	Reactive oxygen and nitrogen species
AOP	Total antioxidant potential
MDA	Malondialdehyde
Osi	Oxidative stress index
4HNE	4-Hydroxy-2-nonenal
3NIT	3-Nitrotyrosine

IHC	Immunohistochemistry
ABTS	2, 20-Azinobis-3-ethylbenzothiazoline-6-sulfonic acid solution
M2VP	1-methyl-2-vinyl-pyridium trifluoromethane
GSH	Reduced glutathione
GSSG	Oxidized glutathione
MSU VDL	Michigan State University Veterinary Diagnostic Laboratory
H&E	Hematoxylin and eosin
CV	Coefficient of variation
CF	Crude fat
Q1	Quintile 1
Q5	Quintile 5

CHAPTER 1

Introduction

Copper nutrition

Copper is required as a co-factor in hundreds of enzymatic reactions in mammalian species. These copper-dependent enzymes are essential in everything from red blood cell production, energy manufacturing, hormone formation, and collagen synthesis to protective mechanisms such as reactive oxygen defense via super oxide dismutase.[1,2] Cytochrome c oxidase (CCO), for example, is a copper-dependent enzyme that is responsible for the terminal electron transfer and hence energy production in all tissues of the body.[3] CCO is also a major component of the oxidative burst in polymorphonuclear cells. The oxidative burst is an important part of the innate immune system and marginal copper deficiency has been found to decrease the pathogen killing capabilities of polymorphonuclear cells.[4] Hence, copper is not only essential for life, but it is also necessary for optimal animal health and production.

Copper has poor dietary availability in ruminant animals.[3,5] Consider that approximately 70-85% of dietary copper is absorbed by the pre-ruminant calf in contrast to <1-10% in adult cattle.[3,6] The rumen is a reductive environment and thus a diet rich in molybdenum and sulfur will bind copper forming thiomolybdate complexes. Thiomolybdates in the rumen anchor copper to organic matter so that it passes unabsorbed in the feces.[7] Additionally, a diet high in iron or manganese have also been found to decrease copper absorption in ruminants.[6,8] High amounts of undigested fiber in the rumen can reduce the bioavailability of copper as well. Moreover, one study found that fiber source or fiber flow rate affects absorption of copper.[9]

Copper absorption is affected by the type of copper supplement utilized in the diet. The types of copper supplements can be broken down into two major classes: organic and inorganic. The most commonly fed inorganic copper sources include copper oxide, copper carbonate, hydroxy copper (dicopper chloride trihydroxide), and copper sulfate. Copper oxide is less available relative to copper carbonate and cupric sulfate. [10] Hydroxy sourced copper has been found to be less soluble in the rumen than copper sulfate, but absorption rates vary with dietary ingredients.[9] Organic copper supplements include copper that is complexed with various amino acids or carbohydrates. Organic forms of copper supplements have varying rates in absorption in the face of antagonists in comparison to cupric sulfate.[3]

Dietary copper concentration varies depending on type and source of ingredients and therefore can be difficult to predict.[1,10] For example, the copper content of wheat mill analyzed at one laboratory ranged from 2-153 ppm. If included at 1.8 kg/head/day, wheat mill could contribute 0.2-12 ppm of copper.[11] An adult lactating cow requires approximately 15.7 ppm copper in the complete diet.[10] Therefore the amount of supplementation required in addition to the basal diet in order to meet copper requirements can range greatly. In fact, copper and other minerals are commonly supplemented in the diet of confined dairy cattle without regard to copper concentration in the basal diet.[12] It has been hypothesized that this has led to supplementation of copper greater than nutritional requirements and this has subsequently led to increased hepatic copper concentrations in dairy cows.[13,14]

Hypocuprosis, or copper deficiency, in ruminants can develop in response to a diet that lacks adequate copper concentrations, elevated concentrations of antagonists or a combination of the two. Thiomolybdates, or molybdenum that is bound to 1-4 sulfur atoms, are powerful copper antagonists that can lead to copper deficiency. Thiomolybdate complexes are not only able to

prevent copper from being absorbed, but they can also be absorbed themselves and bind copper in circulation and in the liver, hastening copper's excretion into bile.[15] Copper deficiency has vague but comprehensive clinical signs. Indeed, disorders affecting almost every organ system in the body have been attributed to hypocuprosis. One of the most classic signs of hypocuprosis in cattle is hypopigmentation of the hair especially around the eyes. In addition to hypopigmentation, copper deficiency can result in rough hair coats and steely wool, or decreased crimping, in sheep. Clinical signs of hypocuprosis in lambs include ataxia which is commonly referred to as swayback and is caused by demyelination of the cerebral cortex.[16 17] In growing ruminants, hypocruprosis may result in rickets-like bone lesions and osteochondrosis with gross defects in articular cartilage. Other clinical signs include anemia, diarrhea, increased susceptibility to infection, and infertility.[3]

Ruminants are likely more susceptible to copper toxicosis than other species as an adaptation to grazing copper-deficient pastures which may contain antagonistic minerals. Ruminants have poor control over copper absorption, store excess copper in their liver and do not excrete copper into bile sufficiently to modulate excessive hepatic copper concentrations relative to other species.[18] Copper toxicosis can occur after a large dose of copper has been given often times parentally from a dosing error. Copper toxicosis also occurs when excessive copper is supplied in the diet and hepatic copper reserves are overwhelmed.[3] This phenomenon occurs more often in sheep than cattle due to their low tolerance for hepatic copper accumulation.[19] In cattle, copper toxicosis is characterized by anorexia, icterus, centrilobular liver necrosis, and tubular nephrosis.[20]

Biomarkers for copper status

Plasma copper concentrations are poorly correlated to hepatic concentrations and therefore other biomarkers are often used in order to characterize the copper status of an animal or herd.[21] Biomarkers for copper commonly include ceruloplasmin and super oxide dismutase. Ceruloplasmin in an acute phase protein that is estimated to bind >80% of plasma copper in humans.[22] Ceruloplasmin plays a role in iron regulation and has antioxidant properties in the blood.[23] Depressed ceruloplasmin activity can occur in copper deficient diets and activity can be increased with copper supplementation. Since ceruloplasmin is a positive acute phase protein it is increased, and so are copper concentrations, in serum during acute and chronic inflammatory states irrespective of hepatic copper concentrations. Conversely, ceruloplasmin can be decreased as a result of liver disease and protein losing nephrosis.[22] In dairy cows, stage of lactation has been known to affect the plasma levels of ceruloplasmin.[24] For these reasons, ceruloplasmin is only a useful biomarker for copper status in cattle to confirm nutritional deficiency and subsequent response to supplementation on a herd level basis.

Super oxide dismutase (SOD) is another potential biomarker for copper status in circulation. SOD is located in the cytosol of most cells and serves as one of the body's primary defendants against oxygen free radicals. The SOD activity in erythrocytes tends to parallel that of certain copper-containing tissues including liver and is not altered by age or gender.[22] However, SOD activity can be affected by oxidative stress and therefore is not an ideal biomarker for copper status.[25]

Additionally, plasma diamine oxidase, a copper containing enzyme has been found to positively correlate with copper deficiency. However, this has not been thoroughly investigated and concentrations can be affected by kidney disease, intestinal disease and pregnancy.[8]

Biomarkers for copper induced hepatic injury

Hepatocyte leakage enzymes have also been investigated as potential biomarkers for hepatic copper status. Hepatic copper accumulation can damage hepatocytes causing them to release certain enzymes into circulation. Hepatocyte leakage enzymes are commonly utilized to diagnose copper accumulation hepatopathies in sheep, dogs and humans.[19,26,27] However, many of the serum enzyme activities are not specific for liver disease and can originate from other cells in the body. So it is common to analyze multiple enzymes at once to increase diagnostic specificity. In sheep, one study found that serum glutamate dehydrogenase (GLDH) activity was the most sensitive liver enzyme for detection of chronic copper toxicosis.[19] GLDH is a mitochondrial enzyme found nearly exclusively in hepatocytes. Elevations in serum GLDH activity usually indicate acute hepatocellular damage. In contrast, GLDH serum activity can be normal or low during chronic hepatic disease that does not involve active cellular death or disruption. Current clinical perception suggests elevated GLDH serum activity is often associated with high liver copper concentrations in cattle.[28-30]

Other studies have found correlations with excessive hepatic copper accumulation in sheep with increases in serum γ-Glutamyltransferase (GGT) and sorbitol dehydrogenase (SDH).[31] GGT is elevated in adult cattle in response to chronic liver disease, especially when the biliary system is involved. Normally, GGT is found primarily in the biliary tract epithelium and elevated serum activity indicates biliary damage or hyperplasia. GGT is also found in the kidney, pancreas,

mammary gland, lung, and other duct epithelium, so while it is a sensitive indicator of biliary disease, elevated serum GGT activity is not a specific indicator of liver damage.[28,29,32] SDH is a cytosolic enzyme located almost exclusively in hepatocytes. Thus, elevations in its serum activity are specific indicators of liver disease and can detect acute hepatocellular necrosis. However, the enzyme protein is relatively unstable in serum and thus elevations in serum SDH activity are short-term indicators of hepatocellular damage.[28,33] Aspartate aminotransferase (AST) is both a cytosolic and mitochondrial enzyme found in liver, muscle, and heart. Therefore, elevations in its serum activity are not specific indications of liver damage.[28-30]

Bile acids (BA) are an important component of the intestinal process of fat digestion and absorption. They are synthesized in hepatocytes and secreted into the intestine as a component of bile. They are reabsorbed from the intestine, flowing back to the liver in portal blood. Under conditions of normal hepatocellular function, bile acids are efficiently extracted from portal blood such that bile acid concentrations in peripheral blood are normally very low. When hepatocellular function is impaired, the efficiency of extraction from portal blood is reduced, allowing concentrations of bile acids in peripheral blood to increase. This makes peripheral bile acid concentration a particularly good indicator of hepatocellular function. This is in contrast to leakage enzymes that are more related to cellular death or damage than functional capacity.[29]

The most reliable indicator of copper status is the liver biopsy.[34] Percutaneous liver biopsies are easy to collect on farm and they pose little health risks to cattle.[35] Liver biopsies for qualitative and quantitative copper assessment for the diagnosis of copper accumulation disorders are routinely used in humans, dogs, and cattle.[36-38] Qualitative analysis utilizing copper-specific stains is beneficial because it demonstrates tissue distribution and can be used to estimate copper concentration.[39] However, copper-specific stains are dependent on cellular localization of

copper. Therefore quantitative analysis of copper in hepatic tissue is considered to be more accurate and has the benefit of analyzing other minerals simultaneously.[35] Various methods for quantitative and qualitative copper analysis on liver tissue are discussed below.

Copper quantification

ICP-MS

Inductively coupled plasma mass spectrometry (ICP-MS) combines high temperature with a mass spectrometer for quantitative analysis of copper and other minerals. Argon is discharged through an ICP torch (6,000-10,000 K) which ionizes a sample that can then be separated and quantified by the mass spectrometer. It has a low detection limit in the parts per trillion, distinguishes between isotopes and measures multiple metals at once. However, it converts atoms into cations so quantification of halogens is difficult. Additionally, the temperature of the ICP torch will destroy all molecular structure which can be a disadvantage of the traditional ICP-MS.[40]

AA

Atomic absorption is a spectro-analytical procedure for quantitative analysis of elements using absorption of optical radiation by free atoms. Atomic absorption utilizes the Beer-Lambert Law to quantify minerals. Briefly, each metal absorbs a discrete, characteristic wavelength when its electrons are promoted to the excited state. The absorbance detected from the emission spectra of each element can then be converted to a concentration by using the Beer-Lambert Law.[41,42]

Rhodanine staining

Rhodanine staining is a technique that attempts to quantify the relative concentration of copper in fixed tissue. Rhodanine specifically stains copper-filled lysosomes.[43] As discussed in more detail later, lysosomes become an important storage mechanism for copper once cytosolic protein storage mechanisms have become overwhelmed. Therefore, rhodanine staining is only significantly visible (1 copper granule per 10x field) once hepatic copper concentrations reach a certain threshold. Rhodanine stained granules then increase in abundance with increasing hepatic copper concentrations. Thus rhodanine qualitative scoring mechanisms have been developed in the dog because canine liver biopsies are commonly evaluated histologically for liver pathologies. These qualitative scores were developed by correlating the relative amount of rhadanine stained copper granules in the centrilobular region of canine hepatic tissue with laboratory quantification of copper in liver tissue by atomic absorption.[39] This study resulted in a scoring system for dogs so that copper concentrations could be conveniently predicted from rhodanine stained slides.[44] The canine rhodanine scoring system has been summarized in table 1.1.

Rhodanine Score	Stain Distribution	MSU VDL Estimated Copper Concentration
0	<1 granule per 10x field	<400 µg/g
1	<2 granules per 10x field	450-650 µg/g
2	Small to moderate amount of granule in <50% of zone 3 hepatocytes	600-900 µg/g
3	Moderate to large amounts of granules in 50-75% of zone 3 hepatocytes	900-1100 µg/g
4	Moderate to large amounts of granules in >75% of zone 3 hepatocytes	1200-1800 µg/g
5	Panlobular granule distribution	>2,000 µg/g

Table 1.1: Rhodanine scoring system and corresponding estimation of hepatic copper concentrations that is used at Michigan State University.

Copper cellular storage, trafficking and metabolism

Research in trafficking and storage of copper has increased over the last decade to aid in the understanding of copper-storage related disorders in humans. These disorders include Wilson's and Menke's disease. Wilson's disease is caused from a genetic mutation in the ATP7B gene which causes a pathologic increase in hepatic copper concentration.[2,45] Menke's disease, on the other hand, is caused by a mutation in the ATP7A gene which leads to copper deficiency as a result of impaired copper absorption from the intestines.[2]

The Ctr1 copper transporter family is present in a multitude of organisms including mammals, yeast and even plants. Ctr1 serves to transport cuprous copper across outer membranes into

cells. It has a well-conserved motif across organisms. Its N-terminus contains a methionine-rich motif and conserved cysteine and histidine residues at the C-terminus.[46] Ctr1 is located on the basal aspect of the hepatocyte. Once in the cytosol, cuprous copper is bound to chaperone proteins. Chaperone proteins include copper chaperone for super oxide dismustase (CCS) which transports copper to SOD. Antioxidant 1 copper chaperone (Atox1) delivers copper to the ATPases ATP7A and ATP7B to be ultimately incoorporated into ceruloplasmin. Cyclooxygenase (COX17) transports copper to cytochrome C oxidase (CCO).[44,46] Glutathione serves as an antioxidant in the cell by binding copper. Additionally, copper bound glutathione has also been shown to have its own redox potential.[47]

The trans-golgi network (TGN) contains both APT7A and ATP7B proteins. ATP7B transports 6 copper atoms onto feroidase ceruloplasmin, the main copper transport protein in circulation. When hepatic copper concentrations increase above basal levels, ATP7B shifts copper transport into lysosomes or into bile through exocytosis.[48] ATP7A and ATP7B are responsible for exporting copper into blood.[49]

Metallothionein is the primary protein that functions to store and regulate intracellular copper. Additionally, it serves as a major antioxidant within the cell due to its cysteine rich structure and thus high metal binding capacity. Metallothionein binds mono and bivalent transition metals with high affinity by forming polynuclear metal-thiolate clusters.[50] These diamondoid-like cages form clusters which do not allow opportunity for free copper atoms to occur until copper storage mechanisms become overwhelmed.[51,52] Cattle have a relatively small concentration of metallothionein in hepatocytes in comparison to other species such as pigs. Additionally, copper is a poor promoter of metallothionein synthesis in comparison to zinc in cattle.[53]

When hepatocellular copper concentrations reach a critical concentration, copper storage proteins become contained within lysosomes to prevent oxidative damage in the cell.[54] Copper bound to metallothionein likely enters the lysosome through autophagy.[55] Lysosome storage of copper is likely important in cattle considering as hepatic copper concentrations increase, mRNA expression of ATP7A deceases.[56]

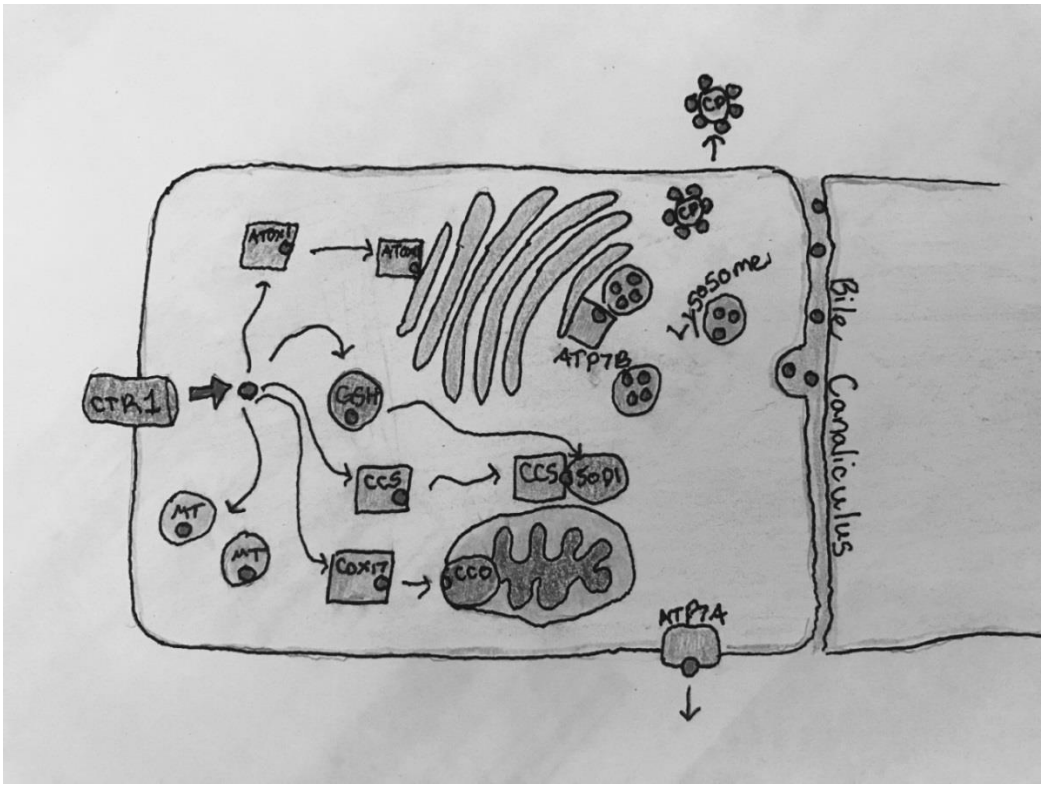

Figure 1.1: Image of copper trafficking and storage in the hepatocyte adapted from Dirksen and Fieten, 2017.

Oxidative stress

Free radicals occur when atoms have an unpaired electron and are therefore unstable. Free oxygen radicals are created as a result of normal energy production such as electron transfer in the mitochondria for production of ATP.[57] Free radicals are necessary for normal processes such as cellular defense, cell differentiation, and protein phosphorylation.[58] In addition to oxygen

radicals, nitrogen radicals, and hydrogen peroxide cause damage by donating or taking electrons from other molecules. Collectively, these are referred to as reactive oxygen and nitrogen species (RONS). Excessive production of RONS can occur from cellular defense against pathogens. Free metals, particularly copper and iron, due to their redox potential are able to create radicals through the Fenton reaction. Oxidative stress can occur when RONS production is greater than antioxidant potential. Antioxidants are molecules that quench radicals by accepting or donating electrons. Antioxidants come in many forms such as peptides like glutathione, vitamins such as vitamin C, vitamin E and beta-carotene, and enzymes such as super oxide dismutase.[59]

Measuring oxidative stress

There are a variety of ways in which to study and measure oxidative stress in cattle. RONS can be measured directly or the products of damage from RONS such as lipid peroxides. Commercially available RONS kits measure hydrogen peroxide, peroxyl radicals, nitric oxide, and peroxynitrite anions in plasma, serum, urine, milk, tissue, and cell lysates.[60] Oxidation products such as lipid peroxides like malondialdehyde (MDA) can be quantified by utilizing commercially available kits.[61] Lipid peroxide products are not only a product of oxidative stress, but they can actually spread, behaving like RONS themselves by oxidizing other lipids and proteins.[62] Lipid peroxidation products like MDA have been commonly used in oxidative stress research, however, they should only be used in consideration with other evidence of oxidative stress. This is because a portion of oxidized lipids measured do not occur in vivo and the results are assay-dependent.[58] Other lipid peroxidation products such as 4-hydroxy-2-nonenal (4HNE) are commonly measured products of oxidative stress. Immunohistochemistry (IHC) staining for 4HNE has been widely used in human medicine for a wide variety of diseases from Alzheimer's to breast cancer. Immunohistochemistry staining for 4HNE utilizes antibodies that bind to the

4HNE-histidine moiety by 3,3'-diaminobenzidine reaction generating a brownish color which can then be visualized by light microscopy. Although this process can be subject to the same assay variables as MDA, the utilization of negative controls aids the researcher in discerning between real results and assay anomalies.[62] Additionally, IHC can be utilized to differentiate which cell types may be more affected than others.

Oxidative damage from RONS is dependent upon the concentration of antioxidant potential (AOP) available to quench them. Concentration of AOP is dependent upon the amount of antioxidants in the diet and rates of utilization and excretion from the body. If measuring RONS in an attempt to characterize oxidative stress it is therefore necessary to quantify AOP as well. AOP can be quantified in samples by standardizing the reduction capacity of Trolox (synthetic vitamin E analog) in 2, 20-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) solution.[63] RONS and AOP can then be used to create a ratio, or oxidative stress index (Osi).[64]

Ratios of non-oxidized to oxidized products can also be measured such as the ratio of reduced to oxidized glutathione (GSH-GSSG). Glutathione itself is an important antioxidant and is also a substrate for glutathione peroxidase, another antioxidant. Low concentrations of glutathione are associated with poor animal health.[58] Additionally, in response to oxidative stress the GSH-GSSG decreases which has been demonstrated in humans with hepatitis.[65]

The field of oxidative stress in dairy cattle medicine is growing rapidly. It has been shown that oxidative stress is linked to inflammatory dysfunction during the transition period. Increased oxidative stress during the transition period occurs from increased cellular respiration from increased energy needs as well as increased β-oxidation in the liver to fuel the copious milk production. Health problems occur when RONS production overwhelms AOP.[66] Increased

oxidative stress in dairy cattle has been linked with liver dysfunction in cows with severe fatty liver degeneration.[67] Oxidative stress is associated with severe mastitis and decreased fertility.[68] Moreover, oxidative stress is increased in cows with ketosis in comparison with those that are not ketotic.[69]

Copper plays an interesting role in the study of oxidative stress because of its role in antioxidant enzymes such as superoxide dismutase, but also its function as a pro-oxidant transition metal. Moderate copper supplementation in cattle has resulted in increased RONS and oxidative stress products both systemically and locally within the liver.[70,71] Abuelo, et al., 2016 found positive correlations of serum copper concentrations with both RONS and AOP.[72] This duality of copper highlights the need for improved reference ranges for hepatic copper concentrations that allow for sufficient antioxidant defenses, but do not increase to concentrations that actually cause oxidative damage.

Copper accumulation and cattle health

There has been increasing concerns that hepatic copper concentrations are increasing in dairy cows to levels that are not only in excess of what is required, but are actually detrimental to animal health.[13,73] These reports demonstrate hepatic copper concentrations found in dairy cows are within or above the super nutritional ranges and are nearing levels where copper toxicosis is a risk.[74] These high hepatic copper concentrations are the result of supplementing copper in the diet above NRC recommendations without evidence of significant dietary antagonists.[75] Moreover, our laboratory has received samples from herds and flocks with concerns that their cattle and sheep's high hepatic copper concentrations are predisposing them to health disorders. These anecdotal reports have come from multiple states, include sheep and cattle and affect

animals ranging in age from 4 month old calves to adult transition cows. Analysis of liver samples submitted from these farms found hepatic copper concentrations nearing toxic levels (>1,400 µg/g in cattle and >800 µg/g in sheep). However, none of these animals examined had elevated liver enzyme activity, icterus or any other signs of hepatopathies associated with copper toxicosis. Instead, these animals experienced increased morbidity and mortality from diseases like mastitis and pneumonia. Thus the question became whether or not high hepatic copper concentrations could cause subclinical liver disease and subsequently increase disease risk through immune dysfunction. Our laboratory sought to investigate the relationship between super nutritional hepatic copper concentrations and liver health in order to better advise our clients on how to best improve the health of their animals.

Hypothesis

The hypothesis of this research is that super nutritional hepatic copper concentrations will result in damage and dysfunction to bovine hepatocytes. The specific aims for this study include investigating the association between hepatic copper concentrations and structural damage to hepatocytes. This will be evaluated by examining histological slides of liver sections for signs of inflammation, fibrosis, necrosis and abundance of copper-stained granules. Additionally, hepatocyte damage will be investigated in association to hepatic copper concentrations by utilizing serum indicators of hepatocyte damage and dysfunction in the form of liver leakage enzymes and bile acids. Moreover, hepatocyte damage from oxidative stress will be investigated by analyzing systemic and hepatic oxidative stress in relation to elevated hepatic copper concentrations. This will be assessed by stratifying the copper concentrations into quintiles and analyzing oxidative stress variables in the lowest and highest quintiles. If such an association is

found, it will suggest that super nutritional, but sub-toxic hepatic copper concentrations may be a risk factor for disease in dairy cattle.

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CHAPTER 2

Introduction

Copper is sequestered in liver of all animals, and particularly so in ruminants, as an apparent reserve supply to provide for nutritional needs during times of insufficient dietary intake. In excess, copper can cause hepatocellular damage and liver necrosis. Copper toxicosis leading to hepatic necrosis and hemolytic crisis can occur either due to an acute consumption of copper or through chronic accumulation in the liver. This phenomenon has been well defined in sheep. [1-6] Conversely, copper toxicosis in dairy cattle has been rarely documented and is not well defined.

The National Research Council recommends that lactating dairy cattle consume 13.7-15.7 ppm copper in the total diet. [7] However, this recommendation is affected by the amount of antagonistic minerals present in the feed such as molybdenum and sulfate. These complex interactions make appropriate copper supplementation challenging for ruminants. There are concerns that dairy cattle are being fed excessive supplemental copper in order to avoid deficiency and copper toxicosis could be a concern for the industry. [8-11] In support of this, Grace et al. and Kendall et al. report that bovine hepatic copper concentrations have been increasing over time in New Zealand and in the U.K., respectively. [10,11] The range of sufficient hepatic reserves is ill-defined, but the expected upper limit is thought to be between 300 and 500 μg/g dry tissue. [12,13] Accordingly, the possible effects of copper concentrations that are greater than what is nutritionally required, but less than that which causes fulminant toxicosis, are poorly understood.

To survey current hepatic copper concentration in Midwest dairy cattle and retrospectively evaluate changes in these concentrations over time, two studies were conducted. In the first part

of the study, our group set out to investigate if hepatic copper concentrations in Holstein cows were increasing over time by reviewing the records of samples submitted to the Michigan State University Veterinary Diagnostic Laboratory (MSU VDL) over a nine year period. In the second portion of the study, we investigated the current hepatic copper concentrations in Midwest cull dairy cows. We then used histological staining to investigate possible correlations between excessive hepatic copper concentrations and hepatocellular damage.

Materials and methods

Animals

In part 1, the MSU VDL database was used for the retrospective analysis of bovine liver copper concentrations for liver biopsy samples submitted from January 1, 2007 to December 31, 2015. Only adult Holstein cows (≥2 years of age) were included. This database contained trace nutrient mineral results from 782 bovine liver samples that had been submitted for routine analysis during the 9 year period. Clinical histories of the animals tested were unavailable. It is known that a majority of the bovine liver biopsy samples are submitted to MSU VDL for routine screening for trace mineral status, however this does not preclude that diseased animals were also included.

In part 2, an abattoir study was conducted. Liver tissue samples were collected from cull dairy cows at a large abattoir in Western Michigan. This study was exempted by the MSU Animal Care and Use Committee. Similar to the retrospective analysis, liver samples (n=149) were only collected from adult dairy cows. Most of the samples came from Holstein cows, although it is possible that liver was collected from additional dairy breeds as well because hides had been removed prior to sample collection. All cows included in the study originated from a livestock auction in southwest Illinois. No dietary or health history could be collected on the study cohort.

No samples were taken from carcasses that were condemned; however, we recognize that this study cohort may not be representative of the general dairy cattle population because ill, low producing, or infertile cows could have been included.

Sample collection

Liver samples were collected by a trained abattoir worker from the caudal edge of the right liver lobe. Samples were collected from this area of the liver because it is the portion accessible for liver biopsy in live animals. Samples were sectioned into formalin within a few hours of collection. The remaining liver was frozen until it could be analyzed for mineral content.

Sample preparation

Copper content was analyzed in fresh liver. A 1-gram section of tissue was digested overnight in a 95°C oven, using approximately 10 times the dry tissue mass of nitric acid. A separate 2-gram section was dried overnight in a 75°C oven to determine the dry matter fraction and calculate the dried tissue mass. The digested samples were diluted with water to 100 times the tissue mass.

Copper quantitation

Elemental analysis was performed using an Agilent Inductively Coupled Plasma Mass Spectrometer (ICP-MS)[a]. [14] Briefly, 200 µl of each diluted tissue digest and calibration standard was diluted 20-fold with a solution containing 0.5% EDTA and Triton X-100, 1% ammonium hydroxide, 2.0% propanol and 5 ppb of scandium, and 7.5 ppb of germanium, rhodium, indium and bismuth as internal standards. The ICP-MS was tuned to yield a minimum of 7500 cps sensitivity for 1 ppb yttrium (mass 89), less than 1.0% oxide level as determined by the 156/140 mass ratio and less than 2.0% double charged ions as determined by the 70/140 mass ratio.

[a] Agilent Technologies Inc, Santa Clara CA 95051

Elemental concentrations were calibrated using a 5-point linear curve of the analyte-internal standard response ratio. Standards were from Inorganic Ventures[b]. NIST[c] Bovine Liver and Mussel standards were used as controls. Copper concentrations were reported on a dry tissue basis.

Histopathology

Liver sections for histological analysis were allowed to be fully fixed in formalin before being cut and embedded in paraffin wax. Paraffin embedded blocks were cut (5 micrometers) and stained with hematoxylin and eosin (H&E) and rhodanine stains. Histology slides were reviewed and scored by a board certified pathologist (DGS). They were scored based on the amount of inflammation, fibrosis, centrilobular hepatocellular change, and the relative amount of copper stained rhodanine granules. The severity of each variable was scored from 0-2. For inflammation a score of 0 meant that there were no visible inflammatory cells, a score of 1 had a mild inflammatory infiltrate with low numbers of lymphocytes, plasma cells, and histiocytes within the portal region, and sections with a score of 2 had severe inflammation with high numbers of the before mentioned inflammatory cells with the addition of neutrophils within the periportal regions. For fibrosis, a score of zero meant that the section had no fibrosis, a score of 1 meant that the section had mild expansion of the portal areas by dense material fibrosis, and sections with a score of 2 demonstrated severe, bridging fibrosis between portal areas. Centriloboular change was defined by pathologic changes within hepatocytes in the centrilobular areas. A score of zero indicated that there were no pathological signs noted in that region, a score of 1 meant that a section had mild cytoplasmic vacuolation in the centrilobular hepatocytes, and a score of 2 would be given to sections with centrilobular necrosis. Rhodanine stained slides were also

[b] Inorganic Ventures, Christainsburg, VA 24073
[c] National Institute of Standards and Technology, Gaithersburg MD 20899

graded 0-2 with a score of 0 showing no rhodanine stained granules, a score of 1 indicated that there were moderate rhodanine stained granules in less than 50% of centrilobular hepatocytes, and a score of 2 indicated that there were moderate to large amounts of rhodanine stained granules in greater than 50% of the centrilobular hepatocytes.

Statistics

Part 1 was assessed by Spearman correlation (SAS version 6.9). Kruskal-Wallis testing was used for the analysis in part 2 of this study. The median hepatic copper concentration for each histological score within each variable was analyzed. For all analyses, $P < 0.05$ was considered significant. All figures were produced with Graphpad Prism (version 6.07).

Results

In part 1 of our study, the mean hepatic copper concentration was 473 µg/g, the median was 443 µg/g, and concentrations ranged from 3-1963 µg/g. From 2007 to 2015 hepatic copper concentrations decreased ($r^2 = 0.45$, $p < 0.05$) (figure 2.1). However, the mean copper concentration in 2007 was unusually elevated (675.87 µg/g) and it only contained a small sample size (n=23). When data from 2007 was removed from the dataset there was no significant change of hepatic copper concentrations across years ($p = 0.24$). Therefore, this was considered an unimportant finding. Overall, 40% of the MSU VDL liver samples had copper concentrations greater than 500 µg/g, with 10% greater than 800 µg/g, and 4% greater than 1,000 µg/g.

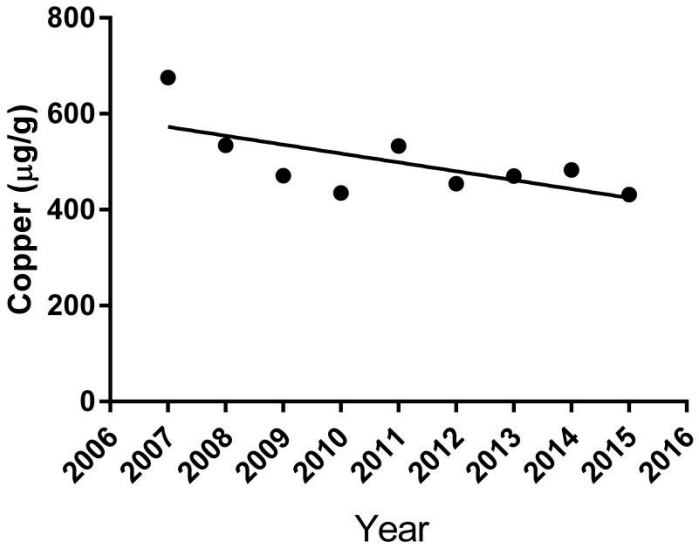

Figure 2.1: Time-trend analysis of the average hepatic copper concentrations (µg/g) for cows in the MSU VDL database from January 1, 2007-December 31, 2015. $R^2 = 0.44$ and $p = 0.049$ for a decreasing trend in mean hepatic copper concentration. 2007 had an inordinately elevated mean hepatic copper concentration with a small number of cows (n = 23).

In part 2 the mean hepatic copper concentrations were 390 µg/g, the median was 400 µg/g and the range was 15-978 µg/g. The mean copper concentration from part 2 was significantly less than part 1 of our study ($p < 0.0002$). In part 2, 26% of samples had copper concentrations greater than 500 µg/g, 4.7% were greater than 800 µg/g, and no measurements were greater than 1,000 µg/g (figure 2.2).

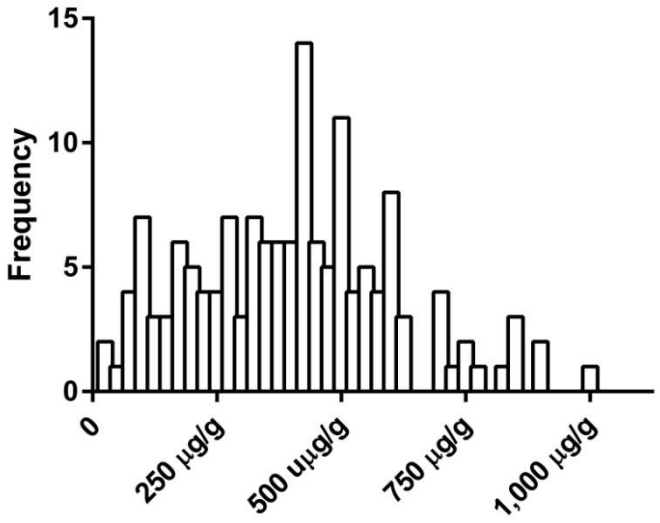

Figure 2.2: Histogram of copper concentrations (µg/g) from abattoir collected samples in part 2 of this study.

Histology findings

In part 2, no evidence of centrilobular hepatocellular necrosis (characteristic finding with copper toxicosis) was apparent from H&E stained sections. Moreover, there was no significant correlation between copper concentration and centrilobular hepatocellular change or with degree of inflammation ($p>0.1$) (figure 2.3c and 2.3b). There was, however, an expected significant correlation between hepatic copper concentration and the relative amount of rhodanine stained granules ($p<0.01$) (figure 2.3d). Additionally, there was a significant correlation between the amount of fibrosis and liver copper concentrations ($p<0.05$) (figure 2.3a). This last finding was considered to be unimportant in relation to copper concentration because fibrosis was peri-portal and therefore unlikely to have been caused by excessive copper accumulation.

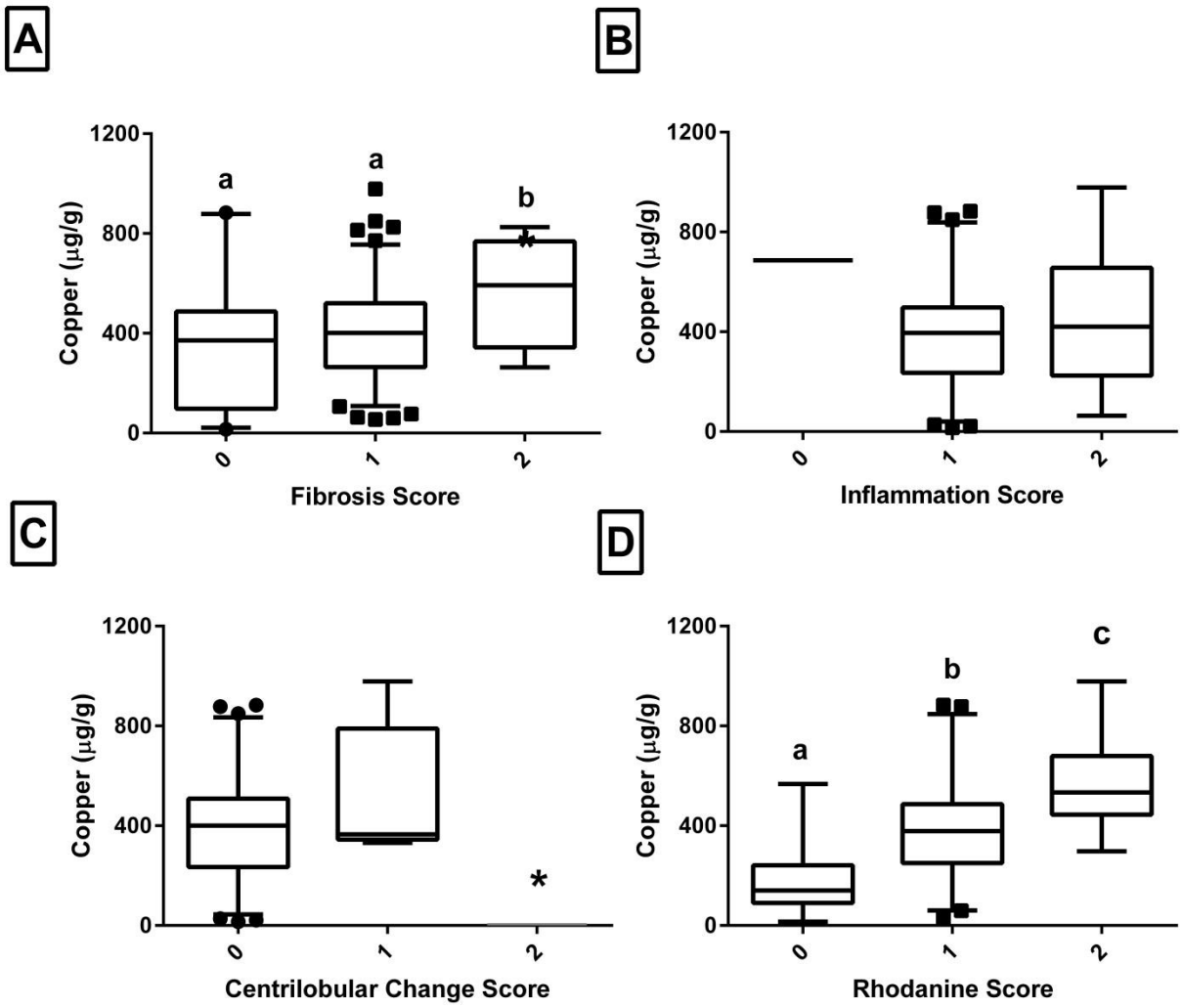

Figure 2.3: Pathologic scoring of A.) Fibrosis, B.) Inflammation, C.) Centrilobular hepatocellular changes, 4.) Rhodanine staining. Statistcal significance at $p < 0.05$ denoted by a, b and c. * = no score provided.

Discussion

According to published reference ranges, the hepatic copper concentrations reported in both part 1 and part 2 of this study would have been considered excessive (table 2.1). This can be difficult to interpret, however, because literature sources do not align on what hepatic copper concentration ranges should be considered adequate, toxic or even deficient. The way in which reference ranges are created is essential when considering which concentrations of copper may

actually harm animal health. For example, current reference ranges reported by the MSU VDL were determined based on data accumulated between 2009 and 2014 on the same instrument. The database included 10,000 diagnostic submissions, which were subdivided by species and life stage such as adult, neonate, or fetus. The expected normal reference ranges were defined as the intervals from the 10th to the 90th percentile of the dataset. Therefore, these reference ranges are based on the distribution of concentrations rather than measurable effects to animal health such as production, oxidative stress, or other biomarkers. Though they serve as a frame of reference for client submitted samples, concentrations above the reference range cannot be taken as detrimental to animal health without supporting evidence. Thus concentrations that are greater than what is required for animal health, but less than what causes overt toxicity, are referred to as 'super nutritional'.

Hepatic Copper Concentrations in the Literature	
Expected Range	**µg/g**
MSU VDL	40-650
AHVLA [10]	<508.37
NUVetNA [10]	<357
Veterinary Clinics of North America [15]	50-300
Buck, 1969 [16]	83.3-500[a]
Toxic	
Tremblay and Baird, 2010 [17]	847-1907[a]
Deficient	
Grace et al., 2010 [18]	<9.5[a]
Marginal Bands	
Suttle, 2010 [12]	6.4-19.2
Suttle, 2016 [19]	341.3-853.3[a]

Table 2.1: A comparison of reference ranges given for bovine hepatic copper concentrations. These are listed on a dry tissue basis. [a] Signifies dry tissue concentrations that have been estimated from wet tissue by dividing the wet weight by 0.3. This is the average dry weight fraction of bovine liver.

Reference ranges for bovine hepatic copper concentrations are often interpreted as a black and white assessment of an animal's copper status. The animal is deemed as either deficient, normal, or super nutritional. Neville Suttle has offered a grey zone for reference ranges that he has referred to as 'marginal bands'.[20] Briefly, marginal bands encompass a range of hepatic copper concentrations for cattle towards the low end nearing deficiency and toward the high end nearing toxicity. The bands represent a range in which one may expect to see clinical changes if supplementation were increased or decreased. When animals in a herd have hepatic copper concentrations that trend toward the lower concentration of the deficiency band, improvement in herd health and production would be expected by increasing copper supplementation.[12] Conversely, animals that have hepatic copper concentrations that trend toward the upper end of the super nutritional marginal band may be at increasing risk for copper toxicosis.[19] Table 2.2 outlines how hepatic copper concentrations from part 2 of the study fell within Suttle's marginal bands. Over half of hepatic copper concentrations from part 2 were greater than what is nutritionally required, or super nutritional, suggesting that these animals were at increased risk for poor health. Indeed, there is evidence that super nutritional concentrations of hepatic copper put cattle at risk for decreased production and increased disease risk.[12,21,22]

	Copper Range (µg/g)	**Percent of Abattoir Tissues**
Deficient	<6.4	0
Deficiency Marginal Band	6.4-19.2	<1
Expected Range	19.3-341.2	40
Super Nutritional Marginal Band	341.3-853.3	58
Super Nutritional	>853.3	2

Table 2.2: Bovine hepatic copper concentrations from part 2 of the study in comparison to Neville Suttle's marginal bands.

In part 2, evidence for super nutritional hepatic copper concentrations was further demonstrated by the correlation of rhodanine-stained section scores with hepatic copper concentrations ($p < 0.01$). Copper has a high pro-oxidant potential and is therefore overwhelmingly protein bound once absorbed from the gastrointestinal tract.[23] Copper remains bound to chaperone proteins, enzymes such as super oxide dismutase, and metallothionein once inside the cell.[24] Metallothionein is the major protein in cells that functions to store and regulate intercellular copper due to its cysteine rich structure which does not allow opportunity for free copper atoms to occur.[25] When hepatocellular copper concentrations reach a critical threshold and normal storage mechanisms are overwhelmed, copper is taken up in lysosomes to prevent oxidative damage in the cell.[26] It is these copper-filled lysosomes that can be visualized by rhodanine staining.[27] Therefore, the presence of these rhodanine stained granules suggests that normal copper storage mechanisms have been inundated and hepatic copper concentrations are in excess. It is not well understood if, or how, copper contained in lysosomes can be utilized.[28] However, it is likely that once rhodanine stained granules are visible, additional copper supplementation would not further benefit animal health.

Excessive copper accumulation is characterized by various histological lesions in other species such as hepatic necrosis in sheep and fibrosis and inflammation in the dog.[1,29] Special attention was shown to centrilobular zones in this study due to the fact that copper toxicity specifically causes necrosis in that portion of the lobule in ruminants.[30] In this study, none of the H&E sections demonstrated centrilobular hepatocellular change such as necrosis despite the fact that nearly 1 in 20 samples had copper concentrations greater than 800 µg/g. Even with the lack of histological evidence, many researchers would have considered these concentrations dangerously close to toxicosis.[8,19] Unlike the canine, there was no correlation found between hepatitis and

copper concentration. Although many H&E sections demonstrated some levels of inflammation, most of the more severe inflammatory samples might be attributed to infectious cause such as salmonellosis. There was a significant correlation with fibrotic lesions and copper concentration. Although this finding was statistically significant, it was not biologically plausible. As previously mentioned, there were no changes in the centrilobular regions of the H&E sections where one would expect the find lesions associated with copper toxicity. Instead, all of the fibrosis occurred in the periportal region. These samples were all taken from cull dairy cattle and periportal fibrosis is a common finding in older cattle. Finally, although secondary copper accumulation disorders can cause lesions in the periportal area, there was no evidence of rhodanine stained granules in this region, which has been found in other species such as cats with cholestatic disorders.[31]

In conclusion, this study demonstrated that Midwest dairy cows had hepatic copper concentrations in excess of what would be expected for well-nourished cattle. Despite these super nutritional hepatic copper concentrations, there were no histologically visible lesions in the architecture of bovine hepatocytes that could be attributed to copper. More research is needed to investigate the effects of hepatic copper concentrations on other measures of liver damage and function, as well as animal health and production.

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CHAPTER 3

Introduction

Liver samples are routinely submitted to the Michigan State University Veterinary Diagnostic Laboratory (MSU VDL) for mineral analysis. Quantitative copper analysis is performed in diagnostic laboratories by either inductively coupled plasma mass spectrometry (ICP-MS), atomic absorption (AA) or inductively couple plasma optical emission spectrometry. Qualitative copper estimation is done by reviewing histological slides of liver stained with "copper-specific stains" such as rhodanine or rubeanic acid. Research has been performed in an attempt to correlate quantitative and qualitative hepatic copper concentrations.[1] Despite the work done in this area, our laboratory at the MSU VDL is occasionally confronted with discrepancies with actual copper concentrations measured by ICP-MS and expected values estimated from rhodanine staining. Thus questions have arisen regarding protocol and analysis accuracy, the effects of sample type and copper heterogeneity in hepatic tissue.

A documented circumstance in which discrepancies arise between qualitative and quantitative copper analysis occurs when different tissue preparations are utilized. The majority of liver samples submitted are fresh or frozen, but occasionally formalin-fixed or paraffin embedded is all that is available. Studies have shown that paraffin-embedded tissues have higher concentrations of copper than their fresh, frozen or formalin fixed counterparts. Despite the variation in results with tissue preparation in these studies, only one study noted that one sample (8% of samples analyzed) would have changed the clinical diagnosis.[2-4]

Variations in hepatic copper concentrations between liver lobes have been well described in cattle and other species including rats and humans.[5-9]Although variations have been found

between copper content in the liver lobes of cattle, the differences have not been clinically significant. Lobular variations in copper content do not explain the discrepancies between qualitative and quantitative copper seen in our laboratory. For example, our laboratory has observed discrepancies in qualitative and quantitative copper concentration within the same piece of liver. Some of those discrepancies would have changed the clinical treatment for that animal depending on which result was used.

The aim of this study was to investigate how extensive the heterogeneity of copper concentrations in hepatic tissue are and how that heterogeneity may be affected by tissue preparation in cattle, goats, and dogs.

Materials and methods

Liver samples investigated included client submitted samples, liver obtained from an abattoir and archived liver in paraffin blocks. Client submitted samples were selected based on previously performed analysis results. Samples were chosen that were recently submitted with copper concentration results that were low, medium or high (n=3). Low copper content was considered to be <400 µg/g copper, medium was 400-1,000 µg/g copper, and high was >1,000 µg/g copper. Abattoir collected samples were chosen based on previously analyzed high copper concentrations (n=4). Archived canine tissues were selected based on copper content that was derived from deparaffinized shavings previously. Forty samples were chosen; 10 with hepatic copper <400 µg/g, 10 with copper 400-1,000 µg/g, 10 with 1,000-2,000 µg/g, and 10 >2,000 µg/g.

Sample preparation

The initial portion of our study was to investigate the variability in copper concentrations in liver. Thus, liver was divided into 3 adjacent strips and then each strip was divided into 5, half-gram sections. One strip was analyzed fresh/frozen, one would be formalin fixed and the third was processed into paraffin blocks and then deparaffinized for analysis. There were then 5 sections analyzed for each tissue process. Formalin fixed sections were stored in formalin for 48 hours in order to allow adequate formalin penetration before analysis. Paraffin embedded samples were first formalin fixed and then embedded in paraffin wax. They were deparaffinized using previously described methods.[2] This previous method was modified to use CitraSolv in lieu of xylene.

Copper content was analyzed in fresh and formalin-fixed liver. A half-gram section of tissue was digested overnight in a 95°C oven, using approximately 10 times the dry tissue mass of nitric acid. A separate 2-gram section was dried overnight in a 75°C oven to determine the dry matter fraction and calculate the dried tissue mass. The digested samples were diluted with water to 100 times the tissue mass.

In a separate analysis copper content was analyzed in homogenized sections of bovine liver. Briefly, strips of liver were divided into 10, half-gram sections from the same sample of liver. These adjacent strips were either analyzed using the above mentioned process or they were homogenized prior to analysis. Batch homogenization consisted of blending the entire strip and then separating out half-gram aliquots for analysis or half-gram sections were first weighed and then homogenized individually prior to analysis.

Copper concentrations were determined on all paraffin embedded archived liver biopsy specimens using modifications of previously described methodology for deparaffinizaton and

analysis.[2] In our study, a more sensitive inductively coupled plasma mass spectrometry (ICP-MS)[a] was used in place of an ultrasonic nebulizer.

Copper quantitation

Elemental analysis was performed using ICP-MS.[10] Briefly, a 200 µl of each diluted tissue digest and calibration standard was diluted 20-fold with a solution containing 0.5% EDTA and Triton X-100, 1% ammonium hydroxide, 2.0% propanol and 5 ppb of scandium, and 7.5 ppb of germanium, rhodium, indium and bismuth as internal standards. The ICP-MS was tuned to yield a minimum of 7500 cps sensitivity for 1 ppb yttrium (mass 89), less than 1.0% oxide level as determined by the 156/140 mass ratio and less than 2.0% double charged ions as determined by the 70/140 mass ratio. Elemental concentrations were calibrated using a 5-point linear curve of the analyte-internal standard response ratio. Standards were from Inorganic Ventures[b]. NIST[c] Bovine Liver, and Mussel standards were used as controls. Copper concentrations were reported on a dry tissue basis.

Histopathology

Liver sections for histological analysis were allowed to be fully fixed in formalin before being cut and embedded in paraffin wax. Paraffin embedded blocks were cut (5 microns) and stained with rhodanine stain. Histology slides were reviewed and scored by board certified pathologists (RCS and DGS). Rhodanine stained slides were then made from those selected blocks and scored based on their relative abundance of rhodanine stained granules using a previously described method for qualitative analysis of copper.[11]

[a] Agilent Technologies Inc, Santa Clara CA 95051
[b] Inorganic Ventures, Christainsburg, VA 24073
[c] National Institute of Standards and Technology, Gaithersburg MD 20899

Rhodanine staining in adjacent tissue sections

A second analysis was performed on formalin-fixed bovine liver (n=4). Formalin fixed sections were chosen based on previously analyzed fresh tissue where copper concentrations were >1,000 µg/g. Formalin-fixed sections were cut into 5 adjacent sections approximately 2 mm thick and paraffin embedded. Sections (n=20) were then rhodanine stained and scored for relative abundance of rhodanine stained granules. Bovine rhodanine stained slides were graded 0-2 using a modified version based on the scoring used for dogs.[11] Briefly, slides with a score of 0 showed no rhodanine stained granules, a score of 1 indicated that there were moderate rhodanine stained granules in less than 50% of centrilobular hepatocytes, and a score of 2 indicated that there were moderate to large amounts of rhodanine stained granules in greater than 50% of the centrilobular hepatocytes.

Statistical analysis

Mean, standard deviation and the coefficient of variation (CV) were calculated for liver copper concentrations. Spearman rank analysis was performed for dog liver copper and rhodanine stain scoring (Graphpad Prism version 6.07).

Results

As copper concentrations in a sample increased, CV also increased. Copper tended to have an increased CV than any other mineral with the exception of sample 1 which had elevated iron (table 3.1).

		Iron		Copper		Zinc		Selenium
Sample 1		**µg/g**		**µg/g**		**µg/g**		**µg/g**
Original Analysis		984.26		9.95		234.04		0.52
Fresh/Frozen		864.27±264.15		10.42±1.42		204.70±66.22		0.46±0.17
CV		30.56		13.62		32.35		37.14
Formalin Fixed		1068.77±230.78		8.65±0.81		218.65±8.70		0.54±0.03
CV		21.59		9.32		3.98		5
Paraffin Embedded		1145.37±66.18		10.48±2.05		248.58±33.26		0.66±0.08
CV		5.78		19.6		13.38		12.34
		Iron		**Copper**		**Zinc**		**Selenium**
Sample 2		**µg/g**		**µg/g**		**µg/g**		**µg/g**
Original Analysis		562.88		717.59		88.46		2.61
Fresh/Frozen		400.56±32.85		485.28±375.87		76.08±10.56		2.53±0.21
CV		8.2		77.45		13.87		8.36
Formalin Fixed		444.84±17.99		625.36±273.02		68.13±10.69		2.85±0.36
CV		4.04		43.66		15.7		12.68
Paraffin Embedded		1142.16±29.19		1799.59±325.21		170.15±5.38		6.4±0.39
CV		2.56		18.07		3.16		6.16
		Iron		**Copper**		**Zinc**		**Selenium**
Sample 3		**µg/g**		**µg/g**		**µg/g**		**µg/g**
Original Analysis		149.93		1544		81.31		4.77
Fresh/Frozen		133.15±29.83		280.03±215.39		57.01±6.72		3.33±0.22
CV		22.41		76.92		11.8		6.58
Formalin Fixed		136.8±9.61		500.02±256.28		63.94±6.36		4.34±0.41
CV		7.02		51.25		9.95		9.47
Paraffin Embedded		208.31±13.19		2138.60±129.92		92.68±4.43		6.27±0.29
CV		6.33		6.08		4.78		4.67

Table 3.1: Mean +/- standard deviation and CV for iron, copper, zinc and selenium in client-submitted samples 1-3.

Batch homogenization greatly decreased CV of copper concentrations. Individual homogenization did not decrease the CV from control demonstrating that the homogenization process itself does not alter copper concentrations table 3.2 and 3.3).

	Mn	Fe	Co	Cu	Zn	Se	Mo
Non-homogenized							
Mean	29.97	2473.42	0.36	1064.47	209.29	2.63	5.32
%CV	5.82	10.26	4.58	42.10	5.46	4.80	4.07
Batch homogenized							
Mean	30.54	2530.75	0.36	1367.79	207.80	2.64	5.35
%CV	3.21	4.84	3.12	4.33	2.95	1.50	1.86

Table 3.2: Mean mineral concentrations and CV in non-homogenized versus batch homogenized liver.

	Mn	Fe	Co	Cu	Zn	Se	Mo
Individually homogenized							
Mean	10.84	138.32	0.21	942.81	150.29	2.44	2.30
%CV	3.96	4.18	4.13	27.31	4.37	3.74	3.79
Batch homogenized							
Mean	11.24	154.27	0.21	1006.31	148.93	2.54	2.31
%CV	1.53	1.43	2.07	1.64	1.94	1.44	1.03

Table 3.3: Mean mineral concentrations and CV in individually homogenized versus batch homogenized liver.

Deparaffinized samples had elevated copper concentrations relative to fresh/frozen and formalin fixed samples. This is likely due to the decrease in tissue when liver is first paraffin embedded and then deparaffinized rather than representing an actual increase in copper content.

Rhodanine scoring of dog livers found good correlation with quantitative analysis of deparaffinized shavings with an $r^2 = 0.7$, however 12 of 40 samples (30% of samples) would have had differing clinical treatment depending on which result was used (figure 3.1).[1] Rhodanine staining of cow livers with high copper concentrations found variability in scores in adjacent tissue sections in 2 of the 4 cows selected (figure 3.2).

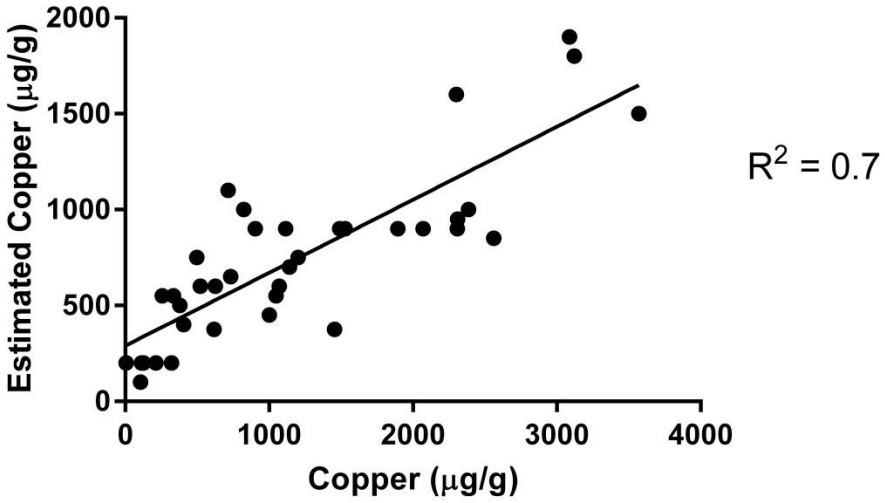

Figure 3.1: Correlation of quantitative copper results from deparaffinized liver shavings with qualitative copper analysis from rhodanine stained slides.

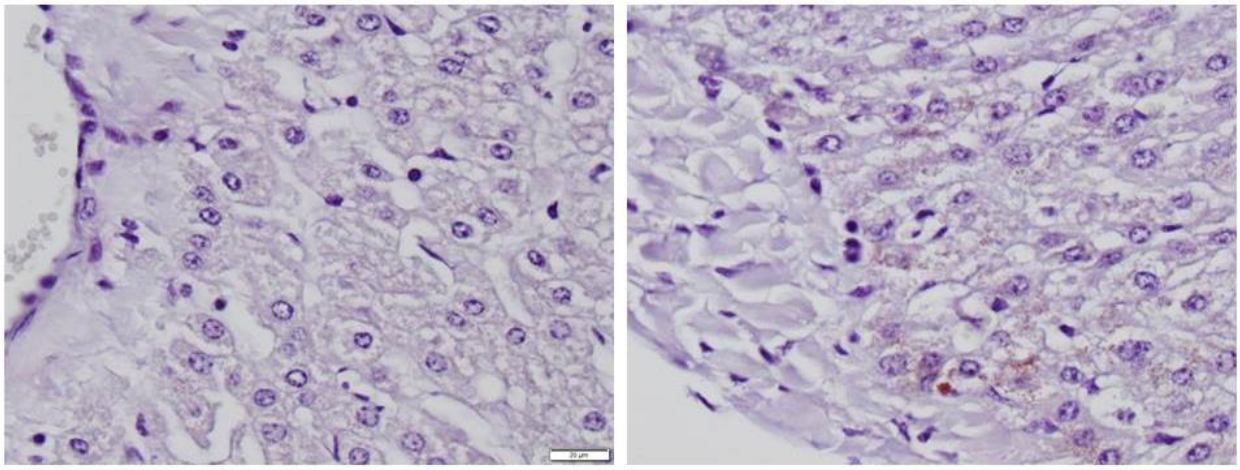

Figure 3.2: Images demonstrating variation in rhodanine scores from the same liver sample. The slide on the left had a rhodanine score of 0 and the slide on the right had a score of 2. The quantitative copper concentration of this liver was 1,123.73 μg/g.

Discussion

Hepatic copper heterogeneity has been demonstrated in other species. Rats on a copper supplemented diet had significantly higher hepatic copper concentrations and greater copper variation throughout hepatic tissue than did rats on an unsupplemented diet.[8] Wilson's disease is

a rare inherited disease in humans where a mutation in the ATP7A gene causes a pathological increase in hepatic copper concentrations. Hepatic copper content in Wilson's disease patients changed 2-3 fold throughout liver tissue. In some patients copper ranged from 880-2100 µg/g in adjacent tissue sections. The same variability in copper concentration throughout the hepatic tissue was also shown with variable rhodanine staining.[9] A similar phenomenon has been shown in dogs with copper associated hepatitis. In one dog with hepatic copper concentrations >2,000 µg/g, copper had a CV of 50%.[3] This variability in copper concentrations in humans and dogs has been attributed to the inflammatory nature of copper accumulation in those species.[3,12] This has largely been considered to be due to severe fibrosis and regenerative nodules. Cattle and goats, however, do not demonstrate the inflammatory reaction to copper accumulation that can be seen in dogs and humans.[13,14] This study found that cattle and goats demonstrate similar variations in copper content when copper concentrations are elevated. This suggests that there is some other factor affecting the distribution of copper in hepatic tissue.

The most striking finding in this study was the magnitude in heterogeneity of copper concentrations as copper content in liver increases. The CV of copper content varied up to 77% in adjacent tissue sections. These variations are noteworthy because the diagnosis would change based on which portion of the liver was analyzed. Copper has been known to vary between lobes for decades, however the variation noted in those studies was not substantial.[6] Other studies have shown that in dairy herds with a high hepatic copper concentrations, there is a larger variation in copper analyzed from biopsies in cows within the herd than there is between cows from a herd on a lower copper status.[15] We hypothesize that the variability seen in herds with an elevated hepatic copper status may actually be due to increased heterogeneity in individual cow livers rather than high variability between cows.

Additionally, rhodanine stained samples demonstrated heterogeneity in scores across adjacent sections in 2 of the 4 bovine livers examined. These tissues had no evidence of inflammation or fibrosis so there is little to account for the variation in rhodanine scores. Quantitative analysis on deparaffinized liver shavings in dogs tended to overestimate copper concentration when compared to rhodanine scores and may have changed treatment decisions in at least 30% of the samples. Deparaffinized samples have been shown to give elevated copper concentrations over fresh or formalin fixed samples.[2,3] Although, there was a good correlation between rhodanine scores and quantitative analysis ($r^2 = 0.7$), shavings from paraffin-embedded samples are not ideal for diagnosis of pathological copper accumulation disorders.

More research is needed to investigate the heterogeneity of copper concentrations in bovine liver. Future studies should include an increased sample size of bovine livers with low, medium and high copper concentrations. Additionally, analysis of entire livers should be included so that blood and bile flow can be taken into consideration. These analyses should include both quantitation and qualitative analysis for comparison of copper concentration between adjacent samples.

This study demonstrated that as hepatic copper concentrations increase, so does the variability of distribution of copper throughout the liver parenchyma. Additionally, this occurs independently of the presence of fibrotic lesions and regenerative nodules. This is an important observation for clinicians who wish to investigate the copper status of herds or individual animals. Based on this study, it is recommended to analyze copper on fresh, frozen or formalin fixed liver only. It is recommended to analyze copper in more than one section of liver. For example, when submitting surgical biopsies, submit 2-3 formalin-fixed biopsies for histological evaluation and 2-3 additional frozen samples for mineral analysis. When taking liver biopsies from cows, it is

recommended to biopsy 12 cows per herd for the most accurate evaluation of copper status.[15] Additionally, if relying solely on rhodanine scoring for hepatic copper analysis, keep in mind that rhodanine staining is also variable and one section may not be representative of the whole liver.

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CHAPTER 4

Introduction

Copper is an essential cofactor in hundreds of enzymatic reactions and is a necessary component of the diet of all species.[1] The digestion of copper in the ruminant animal is complex due to the potential antagonistic interactions of molybdenum, sulfur and iron in the rumen.[2] Therefore, copper is often supplemented in the diets of dairy cattle and over-supplementation can occur.[3] Due to its high redox potential, excessive hepatic copper causes oxidative damage to hepatocellular lipids, proteins, and DNA. This can lead to organelle dysfunction and apoptosis.[4] Abnormal hepatic copper accumulation leading to liver disease occurs in sheep, dogs, and humans.[5-7] If left untreated, excessive hepatic copper accumulation in these species can lead to hepatitis, cirrhosis, hepatic necrosis, and death.[8,9] Excessive hepatic copper accumulation in dairy cattle has been of growing concern in recent years.[10-13] Despite these accounts, the impact of elevated copper concentrations on bovine hepatocytes, short of fulminant toxicosis, is still not well understood.

Damaged hepatocytes release hepatocellular-leakage enzymes into circulation where they can be measured in peripheral blood by routine clinicopathological tests. Activities of these enzymes are therefore commonly used as biomarkers for hepatopathies. Clinicopathological analysis for hepatocellular leakage enzymes is a critical feature in clinical diagnosis for copper accumulation hepatopathies in the above mentioned species.[5,14,15] However, many of the serum enzymes activities that may be elevated in liver disease are not liver-specific and may originate from other cells in the body. For this reason, it is common to measure the activity of multiple serum liver enzymes in one sample to increase the precision of a liver damage diagnosis. Common liver

enzymes measured in dairy cattle include glutamate dehydrogenase (GLDH), γ-glutamyltransferase (GGT), sorbitol dehydrogenase (SDH), and aspartate aminotransferase (AST). In addition to these enzymes, bile acids (BA) are also routinely measured.

The aim of this study was to determine the association between hepatic copper concentrations and evidence of hepatocellular damage in dairy cattle by utilizing serum liver enzyme activities. If such an association is verified, it will suggest high hepatic copper concentrations can result in subclinical liver damage in dairy cows.

Materials and Methods

Sample Collection

Paired blood and liver samples were collected from 100 cull Holstein cows at the time of slaughter. Blood was collected in serum separator tubes[a] during exsanguination. Serum was separated from the clot within an hour of collection (3,000 rpm for 10 minutes) and stored at 4°C until analysis. Liver samples were taken from the caudal right liver lobe by a trained abattoir employee. Liver samples were frozen for later mineral and lipid analysis.

Copper quantitation

Copper content was analyzed in fresh liver utilizing inductively coupled plasma mass spectrometry (ICP-MS)[b] utilizing the protocols set in place at the MSU VDL. A 1-gram section of tissue was digested overnight in a 95°C oven, using approximately 10 times the dry tissue mass of nitric acid. A separate 2-gram section was dried overnight in a 75°C oven to determine the dry matter fraction and calculate the dried tissue mass. The digested samples were diluted with water to 100 times the tissue mass. Elemental analysis was performed using ICP-MS.[16]

[a] BD Vacutainer Serum Separator Tubes, Becton, Dickinson and Company, Franklin Lakes, NJ 07417
[b] Agilent Technologies Inc, Santa Clara CA 95051

Briefly, 200 µl of each diluted tissue digest and calibration standard was diluted 20-fold with a solution containing 0.5% EDTA and Triton X-100, 1% ammonium hydroxide, 2.0% propanol and 5 ppb of scandium, and 7.5 ppb of germanium, rhodium, indium and bismuth as internal standards. The ICP-MS was tuned to yield a minimum of 7500 cps sensitivity for 1 ppb yttrium (mass 89), less than 1.0% oxide level as determined by the 156/140 mass ratio and less than 2.0% double charged ions as determined by the 70/140 mass ratio. Elemental concentrations were calibrated using a 5-point linear curve of the analyte-internal standard response ratio. Standards were from Inorganic Ventures[c]. NIST[d] Bovine Liver and Mussel standards were used as controls. Copper concentrations were reported on a dry tissue basis.

Blood chemistry profiles were performed by Marshfield Laboratories (https://www.marshfieldlabs.org) according to their standard operating procedures. Briefly, all clinicopathological variables were analyzed on the Beckman Coulter AU5800 chemical analyzer[e]. AST[e], GGT[e], SDH[f], GLDH[f], and BA[g] were all analyzed according to the manufacturer's instructions.[17-19]

Crude fat analysis was done by solvent extraction using a modified version of a previously published method.[20] Briefly, 1-gram of liver was fully homogenized with 24 ml of hexane isopropanol (3:2) in 50 ml centrifuge tubes. Next, 12ml of sodium sulfate was added and samples were mixed for 1 minute each. Samples were then centrifuged (3,000 rpm for 5 minutes). The hexane fraction was pipetted off into pre-weighed glass tubes. The sides of the centrifuged tubes were rinsed with 2 ml of hexane, centrifuged and then the remaining hexane fraction was

[c] Inorganic Ventures, Christainsburg, VA 24073
[d] National Institute of Standards and Technology, Gaithersburg, MD 20899
[e] Becker Coulter Diagnostics, Brea, CA 92821
[f] Catachem Inc, Oxford, CT 06478
[g] Diazyme Laboratories Inc, Poway, CA 92064

pipetted into its corresponding glass tube. Once the hexane fully evaporated the glass tubes were weighed again. The percent crude fat was calculated by the following equation:

Crude fat% = (final tube mass-pre-weighed tube mass)/tissue mass x 100

Statistics

Data were analyzed as an exploratory factor analysis (Proc FACTOR, SAS 6.9). Factor extraction was by the PRINCIPAL method and an oblique rotation (OBVARIMAX) was applied. Oblique rotations serve to make factor analysis results easier to interpret and are more realistic than other rotations.[21] Minimum eigenvalues were set at 0.5. A moderate loading was declared at 0.4 to 0.7 and a strong loading was declared at >0.7. Although loadings less than 0.4 are not necessarily unimportant when evaluating factor analyses, those loadings were not considered for interpretation in this study. [22] The matrix of individual Pearson product-moment correlation coefficients (table 4.1) was generated by Proc CORR, SAS 6.9.

Results

	CF	Cu	GGT	AST	GLDH	BA	SDH
CF	1.0000	-0.3053	-0.0981	0.4009	0.1443	0.0439	0.0627
		0.0020	0.3316	<0.0001	0.1521	0.6643	0.5317
Cu	-0.3053	1.0000	0.1211	-0.1302	-0.0025	0.1719	0.1280
	0.0020		0.2301	0.1966	0.9803	0.0872	0.2043
GGT	-0.0981	0.1211	1.0000	0.1371	0.4377	0.1564	0.4023
	0.3316	0.2301		0.1739	<0.0001	0.1201	<0.0001
AST	0.4009	-0.1302	0.1371	1.0000	0.2348	-0.0236	0.2147
	<0.0001	0.1966	0.1739		0.0187	0.8157	0.0320
GLDH	0.1443	-0.0025	0.4377	0.2348	1.0000	0.2161	0.5091
	0.1521	0.9803	<0.0001	0.0187		0.0308	<0.0001
BA	0.0439	0.1719	0.1564	-0.0236	0.2161	1.0000	0.0603
	0.6643	0.0872	0.1201	0.8157	0.0308		0.5512
SDH	0.0633	0.1280	0.4023	0.2147	0.5091	0.0603	1.0000
	0.5317	0.2043	<0.0001	0.0320	<0.0001	0.5512	

Table 4.1: Pearson's correlation of liver leakage enzymes, bile acids and hepatic crude fat for all of the study samples (n=100). The top number is ρ, or the correlation coefficient, and the bottom number is the p value for the correlation of the 2 variables.

The mean hepatic copper concentration was 496.83 µg/g, median was 469.72 µg/g, and the concentrations ranged from 70.56 to 1264.27 µg/g (reference range: 40-650 µg/g). These results were similar to previous abattoir studies of cull dairy cows performed by our group in that hepatic copper concentrations substantially exceed those necessary for nutritional adequacy.[23]

Liver leakage enzyme activity, bile acid, and hepatic crude fat results are shown in table 4.2.

A Pearson's matrix correlation was performed (table 4.1). These correlations were then utilized to produce the exploratory factor analysis for latent variables.

Variable	Mean	Minimum	Reference Range
GLDH (U/L)	23.5	4-80	6-68
SDH (U/L)	19.66	7-72.6	6.6-37.8
GGT (U/L)	28.07	13-68	4-41
BA (µmol/L)	28.12	2-174	0-12
AST (U/L)	91.43	32-394	48-204
Hepatic Crude Fat (%)	7.96	4.44-33.11	3-8

Table 4.2: Summary of serum liver leakage enzyme activity, bile acid concentration and hepatic crude fat percentage in cull Holstein dairy cows (n=100).

The factor analysis resulted in 5 total factors. In factor 1 the variables with relatively high loadings were variables associated with liver health and function (GLDH and SDH) with a moderate loading of GGT (figure 2A). Thus factor 1 represented a "hepatocyte health" factor. Liver copper was not associated with factor 1 with a very low loading. Factor 2 had a relatively high loading of liver crude fat percentage and AST. Factor 2 represented the "hepatic lipidosis" factor (figure 2B). Factor 3 had a very high loading of hepatic copper concentrations and a moderate negative loading for hepatic crude fat (figure 2C). Factor 3 was the copper factor and supports the clinical impression that fatty infiltration reduces hepatic mineral concentrations by dilution of normal parenchyma. Factor 4 had a strong loading for total bile acids with no strong loadings of other variables (figure 2D). Factor 5 was the GGT variable with a strong loading of only GGT (figure 2E).

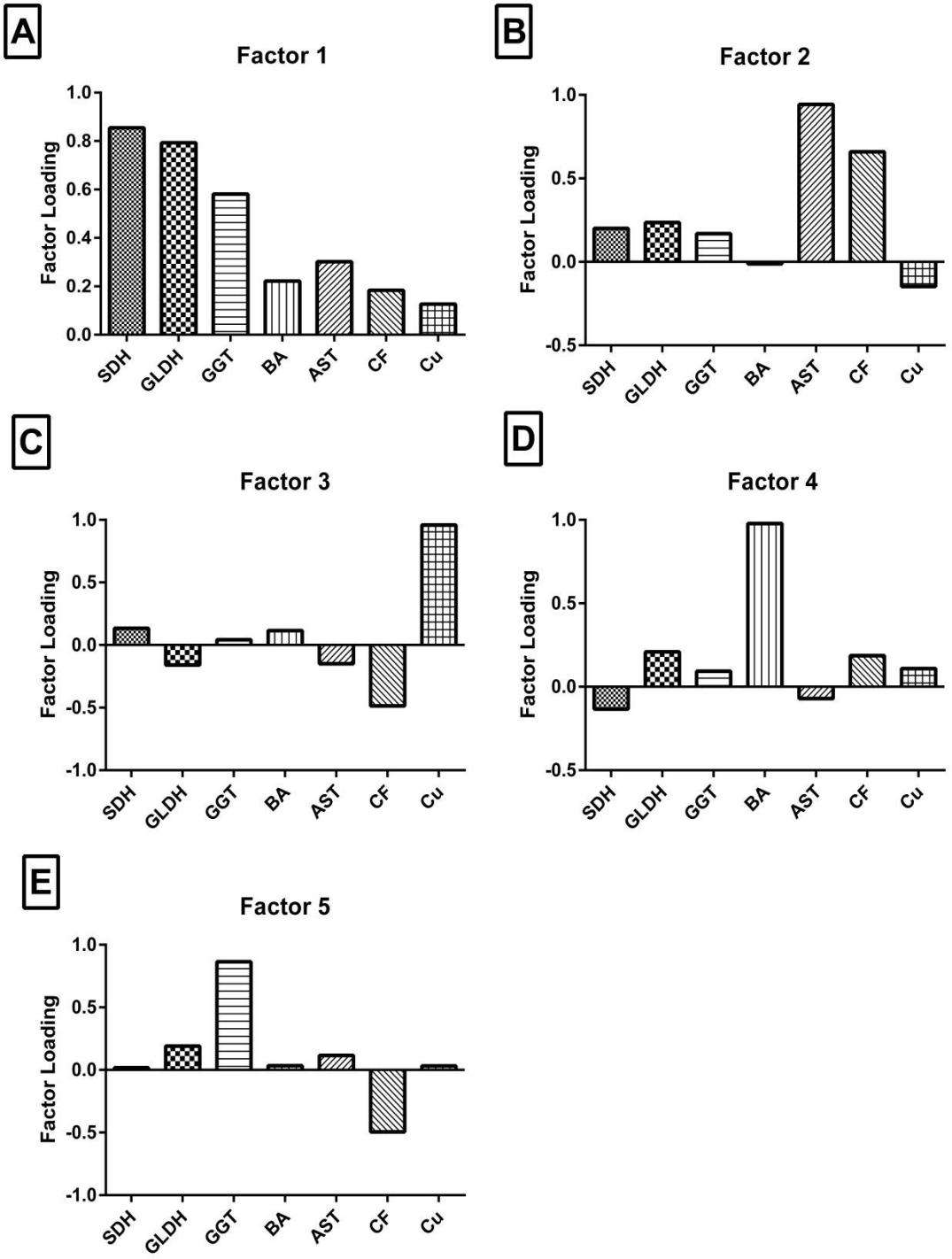

Figure: 4.1: Factor analysis of liver leakage enzymes, bile acids, and hepatic crude fat with an oblique rotation represented in graphic form. A moderate loading was considered to be 0.4-0.7 and a strong loading was considered >0.7-1.0. The latent variables are represented as A.) hepatocyte health factor, B.) hepatic lipidosis factor, C.) copper factor, D.) bile acid factor and E.) GGT factor.

Discussion

Factor analysis is a powerful means of examining and interpreting associations among multivariate data. It is used frequently in social sciences, but less frequently in physical and biological sciences. The correlation matrix of multiple variables forms the basis of the data for factor analysis. The analysis creates unmeasured variables called *factors*. The strength of association between each measured variable and each factor is defined by the *loading* parameter, which can vary between -1 and +1 and is interpreted similarly to a correlation coefficient. A relatively high absolute loading of a measured variable on a given factor indicates the strength of its association, positive or negative, with that factor. Of particular interest in the interpretation of factor analysis is the grouping of measured variables among the factors, because these groupings define the biological nature of the factor.

The factors are sometimes referred to as *latent variables*, suggesting they are phenomena of interest, but are difficult to measure objectively. In social sciences, latent variables could be such things as *emotional health*, which might be defined by an association of measurable variables such as feelings of anxiety, fear of rejection, etc. In the current study we wish to examine the association among multiple variables, all of which are related to hepatic injury or dysfunction. Our expectation is the factors that emerge will represent different aspects, types, or dimensions of hepatic injury or dysfunction. Further, and pursuant to our major objective, we wish to see if hepatic copper concentration is associated with one or more of these dimensions of hepatic injury or dysfunction. Such an association within the study population would imply that increasing copper concentration might be a risk factor for subclinical hepatic disease.

Factor 1 is the hepatocyte health factor with strong loadings of GLDH, SDH, and a moderate loading of GGT. Hepatocyte health is a good example of an unobservable construct within our study that formed through associations with measurable values of liver health.[21] Both GLDH and SDH are found almost exclusively in hepatocytes.[24] Therefore an elevation in their activity in serum is more specific than other leakage enzymes for liver disease. GLDH is a mitochondrial enzyme and elevations in serum GLDH activity usually indicate acute hepatocellular damage. SDH is a cytosolic enzyme with a short serum half-life and elevations in its serum activity are specific indicators of acute hepatocellular necrosis.[24,25] Conversely, GGT primarily originates from biliary epithelial cells and its elevation in serum activity can represent cholestasis.[24] Current clinical perception suggests elevated GLDH serum activity is often associated with high liver copper concentrations in cattle.[25-27] However, this study found no association with elevated hepatic copper concentrations and liver disease as indicated by factor 1. Moreover, liver leakage enzymes in this cohort were primarily within the expected reference ranges.

Factor 2 is the hepatic lipidosis factor. AST is both a cytosolic and mitochondrial enzyme found in liver, muscle, and heart. Thus, elevations in its serum activity are not specific indications of liver damage.[25-27] Factor 2 found an association between AST and hepatic crude fat percentage. Healthy dairy cows have roughly 5% fat in their liver which increases to approximately 8% in the peripartuent period.[28] Hepatic lipidosis (hepatic fat >10%) develops in cattle as a result of negative energy balance, mobilization of body fat stores and an impaired ability for metabolism of lipoproteins.[29,30] The findings in factor 2 were consistent with other investigations that have found AST to be a biomarker for hepatic lipidosis.[29,31,32]

Factor 3 demonstrated a strong loading of hepatic copper and a negative moderate loading of crude liver fat. It is likely that an increase in hepatic fat serves to dilute out liver copper concentrations. Copper is primarily protein bound once inside the cell. Copper is stored in hepatocytes bound to proteins such as metallothionein.[33] As fat increases inside the cell it displaces other molecules such as water and proteins like metallothionein. Thus on a volumetric basis there is less space for copper containing constituents. Similar observations have been found in studies with other species. For example, one study in humans found that patients with non-alcoholic fatty liver disease had 24% less hepatic copper than healthy controls.[34] Note that crude fat is related to both AST and copper in factors 2 and 3, respectively. However, AST and copper are not associated with each other hence their correlations with crude fat must be due to different mechanisms.

Factor 4 has a strong loading of BA. In contrast to liver leakage enzymes, serum BA represent hepatocellular function rather than damage. This is because BA are synthesized by hepatocytes, secreted into the intestines as a component of bile and then reabsorbed by hepatocytes from portal blood. Under conditions of normal hepatocellular function, bile acids are efficiently extracted from blood such that BA concentrations in peripheral blood are normally very low. However, when hepatocellular function is impaired, efficiency of extraction from portal blood is reduced and BA concentrations in peripheral blood increase.[26] Factor 4 had no strong associations with any other variable suggesting that hepatocellular function was not associated with hepatocellular damage, copper concentrations, or crude fat in this cohort.

Factor 5 had a strong loading of GGT and a moderate negative loading of crude fat. GGT is elevated in adult cattle in response to chronic liver disease, especially when the biliary system is

involved. However, GGT is also found in the kidney, pancreas, mammary gland, lung, and other duct epithelium, so while it's a sensitive indicator of biliary disease, elevated serum GGT activity is not a specific indicator of liver damage.[25,26,35] It is unclear why GGT is negatively associated with hepatic CF in this cohort.

In conclusion, this study did not find any serum biomarkers for liver health that are associated with abnormal elevations in hepatic copper concentrations. Therefore, there is no evidence that hepatocellular injury or dysfunction in dairy cows will result from the range of hepatic copper concentrations evaluated in this study. These findings are contrary to previous reports in the literature.[27,35,36] This may be due to the apparent resilience of dairy cattle to copper toxicosis relative to other species such as sheep. This study did find that AST is associated with hepatic lipidosis, as has been previously observed.[30,32] Hepatic lipidosis was also found to be negatively correlated with liver copper concentrations. This is likely due to the dilution of hepatic parenchyma with fat, although other metabolic factors cannot be ruled out.

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CHAPTER 5

Introduction

Copper is an essential element in the diets of all animals. Due to its variable concentration in forages fed to cattle and the antagonistic interactions with other minerals such as molybdenum and sulfur in the rumen. Copper is required for the proper function of 100's of enzymes in the body including those that are involved in red blood cell production, iron metabolism and immune function.[1,2] Insufficient dietary copper may lead to decreased health and growth in cattle.[3] Therefore copper is frequently supplemented in the diet of dairy cattle and over supplementation is an increasing concern.[4,5] Copper is stored in the liver primarily protein bound and is excreted into bile.[6] When copper intake exceeds liver storage and the export capabilities then free copper is more likely to occur. Free copper has pro-oxidant potential and in excess it can lead to increased oxidative stress through the Fenton reaction.[7] Oxidative stress in relation to hepatic copper concentrations in dairy cattle has only recently been investigated.[8,9]

Oxidative stress occurs when reactive oxygen and nitrogen species (RONS) overwhelm the organism's total antioxidant potential (AOP). Oxidative status is a balance and therefore it is important to measure both pro-oxidants and antioxidants when determining the relative level of oxidative stress in an animal. In dairy cattle, the oxidative stress index (Osi) which is the ratio RONS/AOP has been established in order to associate total oxidative stress with disease risk.[10] Lipids are particularly vulnerable to oxidative stress, but proteins and DNA are also susceptible. This can lead to organelle dysfunction and apoptosis.[11] Oxidative stress has been linked to dysfunctional immune and inflammatory responses in dairy cows.[12] Moreover, decreasing oxidative stress through supplementation of antioxidants such as vitamin E and selenium in the

diet has been proven to decrease disease severity in metritis and mastitis in dairy cows.[13] Thus investigating factors that may increase oxidative stress, so they might be ameliorated, could have a positive impact on animal health and production.

Our group has demonstrated that high hepatic copper concentrations occur frequently in dairy cows, but the clinical significance of hepatic copper concentrations in this range is unknown. The pro-oxidant characteristics of metallic copper might be a link between high hepatic copper concentrations and clinical disease due to increased risk for oxidative stress. The objective of this research was to determine if high hepatic copper concentrations were associated with evidence of oxidative stress systemically and locally within hepatic tissue. This study was exempted from review by the MSU Animal Care and Use Committee.

Materials and Methods

Sample Collection

One hundred paired liver and blood samples from cull dairy cows were collected at the time of slaughter at a West Michigan abattoir. Blood samples were collected during exsanguination in EDTA and serum separator tubes[a]. Whole blood from EDTA tubes was first pipetted into 1.5 ml cryo-vial tubes. Tubes for oxidized glutathione contained 10 µl of scavenger, 1-methyl-2-vinyl-pyridium trifluoromethane (M2VP), and were mixed with 100 µl of whole blood. Samples for reduced glutathione received 50 µl of whole blood. Plasma and serum samples were spun down completely (10 minutes 3,000 rpm), and pipetted into cryo-vial tubes. The vials of whole blood, plasma, and serum were flash frozen in liquid nitrogen and then later transferred to a -80° C freezer for storage until analysis.

[a] BD Vacutainer Serum Separator and EDTA Tubes, Becton, Dickinson and Company, Franklin Lakes, NJ 07417

Fresh liver (~200 mg) was placed into cryo-vial tubes. These sections of liver were flash frozen in liquid nitrogen and then stored in a -80° C freezer for storage until analysis. Another section of liver was placed in formalin within an hour of collection. The remaining liver was stored at -4° C until analysis.

Mineral Analysis

Hepatic copper content was determined on fresh tissue. Tissues were sectioned (1 gram) and digested overnight in a 95°C oven, using approximately 10 times the dry tissue mass of nitric acid. A separate 2-gram section was dried overnight in a 75°C oven to determine the dry matter fraction and calculate the dried tissue mass. The digested samples were diluted with water to 100 times the tissue mass. Elemental analysis was performed using an Agilent Inductively Coupled Plasma Mass Spectrometer (ICP-MS)[b]. [14] Briefly, 200 µl of each diluted tissue digest and calibration standard was diluted 20-fold with a solution containing 0.5% EDTA and Triton X-100, 1% ammonium hydroxide, 2.0% propanol, and 5 ppb of scandium, and 7.5 ppb of germanium, rhodium, indium and bismuth as internal standards. The ICP-MS was tuned to yield a minimum of 7500 cps sensitivity for 1 ppb yttrium (mass 89), less than 1.0% oxide level as determined by the 156/140 mass ratio and less than 2.0% double charged ions as determined by the 70/140 mass ratio. Elemental concentrations were calibrated using a 5-point linear curve of the analyte-internal standard response ratio. Standards were from Inorganic Ventures[c]. NIST[d] Bovine Liver and Mussel standards were used as controls. Copper concentrations were reported on a dry tissue basis.

Samples were stratified based on copper concentration and divided into quintiles. Variables associated with oxidative stress were measured in the highest and lowest quintiles (Q5 and Q1).

[b] Agilent Technologies Inc, Santa Clara CA 95051
[c] Inorganic Ventures, Christainsburg, VA 24073
[d] National Institute of Standards and Technology, Gaithersburg MD 20899

These variables included serum total reactive oxygen and nitrogen species (RONS), antioxidant potential (AOP), whole-blood reduced to oxidized glutathione ratio (GSH/GSSG), and liver malondialdehyde (MDA).

Oxidative Stress Analysis

AOP was quantified in serum samples as described previously.[15] Briefly, the AOP of a sample was standardized to the reduction capacity of Trolox (synthetic vitamin E analog) in 2, 20-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) solution.[16] RONS were quantified in serum as a marker of pro-oxidant production using a commercially available assay[e] following the manufacturer's instructions. In brief, free radicals in the sample react with a specific probe that is converted into a fluorescent product. Thus, the fluorescent intensity is proportional to the total RONS content in the sample. Fluorescence of the dichlorofluorescent dye was determined at 480 nm of excitation and 530 nm of emission and a standard dichlorofluorescent dye curve was included to ensure that the dye could be detected at various concentrations. Blank values were subtracted from sample values to eliminate background fluorescence. The reported values represent relative fluorescent units normalized per microliter of sample.[17] Concentrations of RONS and AOP were utilized to make Osi ratio.[10]

The GSH/GSSG was quantified with a commercially available kit[f] as previously described.[18] Briefly, whole blood was aliquoted from EDTA collection tubes within 1 hour of collection and a thiol scavenger (M2VP) was immediately added to the GSSG samples before freezing at −80°C in liquid nitrogen. The GSH/GSSG ratio was determined in the presence of 5-5′-dithiobis (2-nitrobenzoic acid) and NADPH according to the supplied manufacturer's protocol. The

[e] ROS and RNS assay, Cell Biolabs, San Diego, CA 92126
[f] Bioxytech GSH-GSSG-412, OxisResearch, Foster City, CA 94404

change in absorbance at 412 nm over 3 min was measured using a spectrophotometer for both samples and standards (0 to 1.5 m*M* of GSSG). The GSH/GSSG ratio was calculated using a standard curve and the sample reaction rates. The GSH/GSSG ratio was calculated from the following equation: (GSH -2GSSG)/GSSG.

MDA was quantified in liver sections with a commercially available kit[g] as previously described.[19] Briefly; 10 mg of liver tissue was sectioned off and washed in cold PBS. Tissues were then homogenized on ice in lysis buffer, centrifuged (13,000 x g for 10 minutes), and then supernate was separated. Supernate was incubated with TBA reagent at 95° C for 1 hour. Samples were then read in a fluorometric plate reader at 532/553 nm.

Immunohistochemistry

Formalin fixed liver sections in Q1 and Q5 (n=40) were stained with immunohistochemistry (IHC) stains for 4-hydroxynonenal (4HNE) and 3-nitrotyrosine (3NIT) at the Department of Veterinary Pathology, Iowa State University. Tissues were cut into 5 micron sections and placed on glass slides. Slides were baked at 57° C for 30 minutes. Tissues were deparaffinized in xylene twice for 5 minutes. Tissues were then rehydrated in 100% alcohol for 3 minutes 3 times each, in 95% alcohol for 2 minutes twice each and then in 70% alcohol for 3 minutes once each. Tissues were then processed with a commercially available Avidin/Biotin blocking kit[h]. In order to inhibit endogenous peroxidase activity, tissues were incubated in 3% hydrogen peroxide for 2, 10 minute applications followed by 3 ultrapure water rinses. For the antigen retrieval process slides were placed in a plastic Coplin jar containing Citra pH 6 antigen retrieval buffer. They were then microwaved on high (full power) until the surface was bubbling. The Coplin jar was

[g] Lipid Peroxidation Assay Kit, Oxford Biomedical Research, Rochester Hills, MI 48309
[h] Avidin/Biotin Blocking Kit, Cat # 004303, ThermoFischer Scientific, Waltham, MA 02451

then moved to a preheated steamer for 20 minutes. They were then left at room temperature for 20 minutes and rinsed twice with PBS. Next, the slides were blocked with 90% NGS/(Tris/PBS/BSA buffer) for 20 minutes. The primary mouse monoclonal antibody was diluted in Tris/PBS/BSA buffer (4-HNE[i] was diluted 1:50 and 3-NIT[j] was dilution 1:100) and incubated for 2 hours followed by 2x PBS rinses, 5 minute PBS bath, and 2x PBS rinses. Next samples were processed with dilute Multilink 1:80 in Tris/PBS/BSA buffer that was applied for 15 minutes followed by 2 PBS rinses, 5 minute PBS bath, and 2 more PBS rinses. Next dilute Horseradish Peroxidase-Streptavidin1:200 in Tris/ PBS/BSA buffer was applied for 15 minutes followed by 2x PBS rinses, 5 minute PBS bath, and 2x PBS rinses. Subsequently, Nova Red stain was applied for 5 minutes and rinsed 5 times with ultrapure water. Slides were then transferred into ¼ strength Shandon's hematoxylin for 2 minutes, rinsed in ultrapure water 3 times, placed in Scott's tap water for 1 minute and then rinsed 3 times in ultrapure water. Slides were then dehydrated once with 70% alcohol, twice with 95% alcohol, 3 times with 100% alcohol and 3 times in Xylene. Coverslip slides were applied with a non-aqueous mounting media[k] and allowed to dry at room temperature. Formalin fixed liver sections were processed for H&E and Rhodanine staining (n=100).

Histopathology scoring

Rhodanine and IHC stained slides were graded in the same manner on a 0-5 scale. A score of 0 showed no IHC or Rhodanine staining. A score of 1 was a section that had minimal granules in less than 33% of centrilobular hepatocytes. A score of 2 indicated that there were moderate Rhodanine or IHC stained granules in less than 50% of centrilobular hepatocytes, and a score of

[i] Anti-4-Hydroxynonenal Antibody, Abcam, Cambridge, MA 02139
[j] Anti-3-Nitrotyrosine Antibody, Santa Cruz Technology, Dallas, TX 75220
[k] Acrytol Mounting Media, Surgipath, Leica Biosystems, Buffalo Grove, IL 60089

3 indicated that there were moderate to large amounts of Rhodanine or IHC stained granules in greater than 50% of the centrilobular hepatocytes. A score of 4 had staining in >75%of zone 3 hepatocytes and a score of 5 demonstrated panlobular staining.

H&E slides were all observed for signs of centrilobular necrosis and were scored on relative amount of inflammation and fibrosis. For inflammation a score of 0 meant that there were no visible inflammatory cells, a score of 1 had a mild inflammatory infiltrate with low numbers of lymphocytes, plasma cells and histiocytes within the portal region, and sections with a score of 2 had severe inflammation with high numbers of the before mentioned inflammatory cells and neutrophils within the periportal regions. For fibrosis, a score of zero meant that the section had no fibrosis, a score of 1 meant that the section had mild expansion of the portal areas by dense material fibrosis, and sections with a score of 2 demonstrated severe, bridging fibrosis between portal areas.

Statistics

The effect of low (Q1) versus high (Q5) hepatic copper concentrations on serum and hepatic variables was assessed by the Mann-Whitney U test. The association of hepatic copper concentration and histopathological changes evaluated by H&E staining was evaluated by the Kruskal Wallis test. The strength and association of histopathological scores as evaluated by rhodanine staining scores was evaluated by linear regression with Graphpad Prism (version 6.07).

Results

Mean copper concentration of all samples was 496.83 µg/g (range 70.56-1264.27 µg/g, reference: 40-650 µg/g). The mean copper concentration of Q1 (215.29 µg/g) was substantially lower than Q5 (825.36 µg/g) ($p=0.0002$).

No evidence of hepatic necrosis, as would be expected with copper toxicosis, could be demonstrated by the histopathological scoring of H&E stained slides ($p>0.99$). There was minimal evidence of inflammation or fibrosis and their scores had no correlation with copper concentrations. Rhodanine staining score increased in positive association with copper concentrations ($r^2 = 0.483$, $p<0.0001$) (figure 5.1).

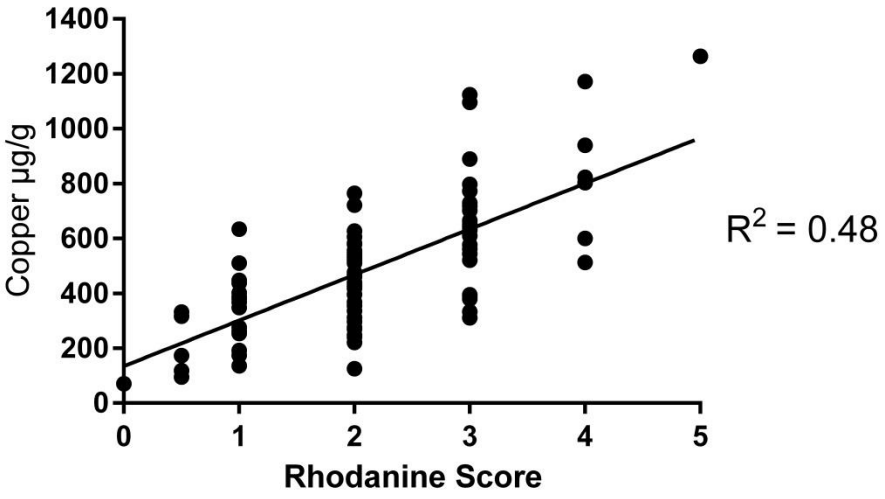

Figure 5.1: Correlation of quantitative copper concentrations µg/g and rhodanine scores.

There was no difference in serum RONS, Osi, whole-blood GSH/GSSG ratio, GSH, or liver MDA that could be demonstrated between Q1 and Q5 ($p>0.54$). AOP was higher in Q5 than in Q1 ($p < 0.01$) (table 5.1).

Variable	Q1	Q5
RONS (RFU/µl)	34.03	39.73
AOP (TE/µl)	16.05	18.75**
Osi	2.17	2.18
GSH (µM)	774.18	725.7
GSH/GSSG	482.01	513
MDA (nmol/mg)	0.036	0.037

Table 5.1: Mean of oxidative stress variables for Q1 and Q5. AOP was higher in Q5 than Q1 ($p < 0.01$). No other variables measured were different between Q1 and Q5. For GSH/GSSG, Q1 has n=15 and Q5 has n=14 because GSSG results that were below detection were discarded. For all other variables, Q1 n= 20 and Q5 n=20.

IHC staining scores in Q5 were significantly higher than Q1 for both 4HNE and 3NIT ($p = 0.0003$ and $p = 0.0058$ respectively)(table 5.2). For both 4HNE and 3NIT, the IHC stain pattern mirrored that of rhodanine in that it originated from the centrilobular area and extended into the lobule to a similar degree (figure 5.2).

Variable		Q1		Q5
4HNE		0.85		2.1***
3NIT		0.2		1.03**

Table 5.2: IHC scores for Q1 and Q5. 4HNE staining scores were higher in Q5 than Q1 ($p < 0.001$) and 3NIT staining scores were higher in Q5 than Q1 ($p < 0.01$).

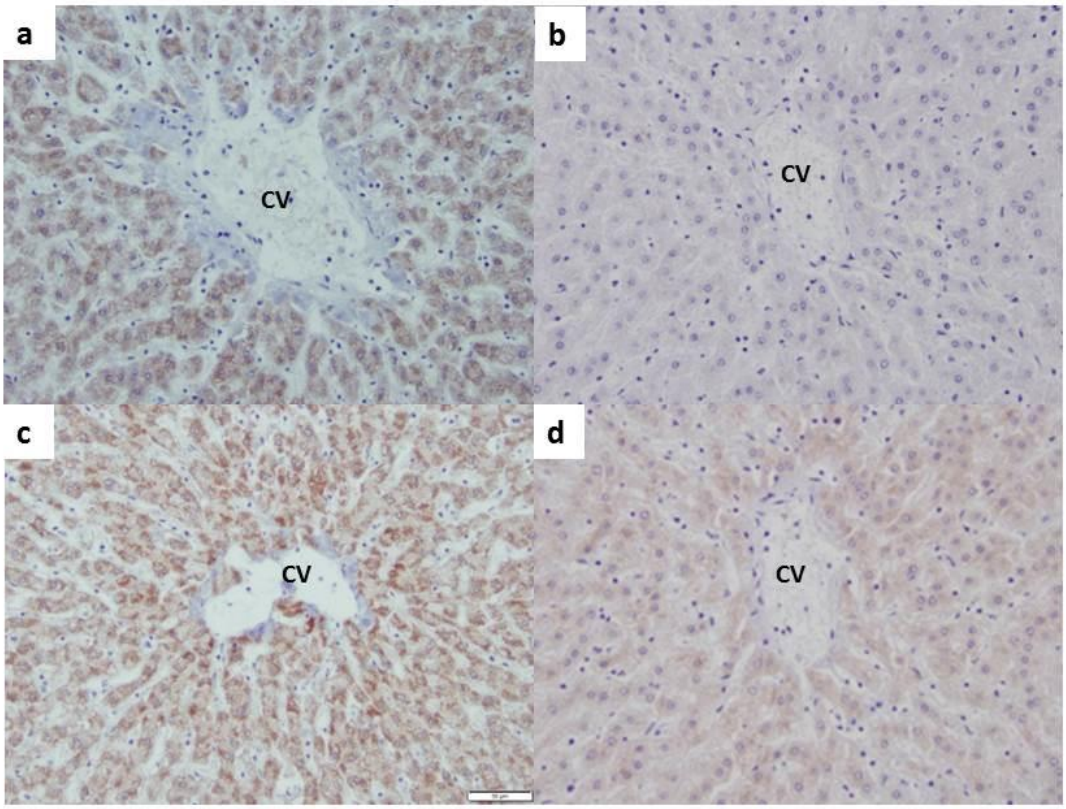

Figure 5.2: The 3NIT (a, b) and 4HNE (c, d) stained slides for two separate samples centered around the central vein (CV) at 20x. Cow 1 (a, c) had a hepatic copper concentration of 1264.27 µg/g. Cow 2 (b, d) had a hepatic copper concentration of 173.24 µg/g (ref. range: 40-650 µg/g).

Discussion

This study demonstrated that hepatic sections in Q5 showed significantly greater oxidative stress as demonstrated by 4HNE and 3NIT IHC staining than those from Q1. Moreover, IHC staining distribution mirrored that of rhodanine staining within hepatic lobules. These findings suggest that excessive, but sub-toxic, hepatic copper accumulation does increase risk of local oxidative stress in the liver. 4HNE stains have been used to study the pathogenesis of copper accumulation in Long-Evans Cinnamon (LEC) rats which serve as a model for Wilson's Disease in humans.

Positive 4HNE staining occurs in LEC rats prior to detectable inflammation by histopathology.[20] This shows that 4HNE staining may be particularly useful in in characterizing subclinical hepatocellular damage caused by copper accumulation in species such as cattle. Other studies have investigated the association between hepatic copper accumulation and oxidative stress in cattle. A Spanish feeding trial demonstrated that copper supplemented calves had increased IHC staining of oxidative stress products (nitrotyrosine and inducible nitric oxide synthase) than controls.[9] Another study which also utilized cull dairy cows found that many samples with high hepatic copper concentrations also had 4HNE staining that mirrored that of rhodanine stained samples.[8] This study did not find a significant difference in 4HNE staining in samples with higher hepatic copper concentrations. This may have been due to their small sample size (n=45) and their decreased variation in hepatic copper concentrations in comparison to this current study.

There was no difference between Q1 and Q5 for RONS, GSH/GSSG, MDA, or Osi. This is likely due to the study population and the uncontrolled variables such as unrelated diseases and shipping stress that can occur in cull dairy cows. RONS was elevated in both groups relative to non-diseased cows at dry off in a previously published report.[17] This suggests that cows in both groups may have been subjected to other diseases or sources of RONS unrelated to hepatic copper accumulation.

AOP was higher in cows with elevated hepatic copper concentrations. The relationship between hepatic copper concentrations and AOP are unknown in this cohort. AOP concentrations can be affected by diet, days since calving, and parity. [23] In addition, AOP tends to be decreased due to disease and in animals with negative energy balance. This is likely due to both increased utilization as a result of increased cellular respiration and fatty acid oxidation in the liver as well

as decreased intake of antioxidants in feed.[21,22] However, age, diet, and health records were unknown in study group. Interestingly, the AOP concentrations in both groups were elevated in relation to previously published data.[16]

There was no difference between Q1 and Q5 demonstrated in the GSH/GSSG ratio. Similar to AOP, concentrations of GSH were elevated relative to concentrations previously reported.[16,23] Samples with GSSG results below detection were excluded from results. Hepatic MDA concentrations were also not different between groups. Study conditions, however, may affect results because lipid peroxidation products are non-specific indicators of oxidative stress and can be affected by many variables. MDA assays have been known to show inconsistent results in dairy cows because very little of the MDA measured is actually produced in vivo and therefore results are heavily determined by assay conditions.[25]

This study showed that cows with increased hepatic copper concentrations had increased oxidative stress in their liver as shown by IHC staining than cows with lower copper concentrations. This is the first study to show that high, but sub-toxic, hepatic copper concentrations cause hepatocyte damage through lipid and protein oxidation. There was no difference in systemic markers of oxidative stress between groups. Systemic markers of oxidative stress are likely not specific for copper accumulation in this cohort. There was a difference in AOP concentrations between quintiles, but the reason for this result was unknown. Future investigations should include feeding trials with varying levels of copper supplementation in order to see if systemic markers for oxidative stress can be accurate measures of hepatic copper accumulation.

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CHAPTER 6

Review

Adequate hepatic copper concentrations in cattle have been estimated to be 100-300 µg/g dry tissue, however, our survey of dairy cows liver samples submitted to the MSU VDL and liver samples collected from 2 separate abattoirs found that mean copper concentrations were elevated above this range.[1] In other species such as humans, dogs and sheep, hepatic copper concentrations elevated above their adequate reference ranges result in hepatic inflammation, fibrosis and necrosis. However, in the cohort of cattle investigated (n = 249), there was no correlation with increasing hepatic copper concentrations and histopathological signs of inflammation, fibrosis, or necrosis.

In humans and dogs with hepatitis it is recommended to collect more than one biopsy for mineral analysis because copper concentrations can vary throughout hepatic parenchyma due to fibrous and regenerative tissue.[2,3] Our group found that there is an increase in the heterogeneity of copper concentrations in the liver of cattle as concentrations increase even in the absence of fibrosis and inflammation. More investigation is needed to investigate the potential mechanisms involved.

Investigations into copper toxicosis in cattle have correlated high hepatic copper concentrations with increased activity of liver leakage enzymes in serum.[4,5] However, in these case reports animals died from fulminant copper toxicosis. Clinical copper toxicosis with hepatic necrosis and hemolysis resulting in death is a relatively rare phenomenon in dairy cattle. Despite this, concerns have been raised that hepatic copper concentrations in cattle are increasing from over supplementation and toxicities may become more frequent.[6-8] In our abattoir study, 58% of the

liver samples were within the super nutritional marginal band and 2% were elevated above the marginal band where there is greater risk for toxicosis. In spite of that, there was no association of hepatic copper concentrations and liver leakage enzymes found.

Our group showed that cows with increased hepatic copper concentrations had increased oxidative stress in their liver as shown by IHC staining. IHC staining of liver sections showed that both an increase in oxidized lipids and proteins were associated with high hepatic copper concentrations. This was the first study to show that high, but sub-toxic, hepatic copper concentrations cause hepatocyte damage through lipid and protein oxidation. There was no difference in systemic markers of oxidative stress between groups. This may have been due to the fact that the study cohort consisted of cull dairy cows from different farms with many unknown variables that may have contributed to systemic concentrations of oxidative stress.

Future directions

In chapter 4 a negative moderate correlation was found between crude fat percentage and hepatic copper concentrations. Although this may have been due to simple dilution of liver parenchyma, metabolic factors could have been involved. For example, a feeding trial that investigated the effects of increasing dietary copper supplementation, and lipid metabolism found that increased dietary copper reduced back fat and serum cholesterol in cattle. [9,10] Engle et al. (2000) hypothesized that copper-induced alterations in lipid metabolism may be due to changes in catecholamine metabolism.[11] A similar study found that adipocytes isolated from subcutaneous fat in steers with increased dietary copper supplementation had increased basal and epinephrine induced lipolytic rates than that of controls.[12] These studies, however, focused on copper intake

and meat quality in beef cows. More studies are needed in the future to investigate the possible relationship between dietary copper and hepatic crude fat in dairy cows.

In chapter 5 no difference was found in systemic oxidative stress levels between the highest and lowest quintiles of hepatic copper concentrations. Oxidative stress could have been affected by a number of variables in this cohort including shipping stress, diseases, varying diets between different farms and heat stress. More controlled feeding trials are needed in the future to investigate whether or not there actually are increased systemic oxidative stress markers in cows with a higher hepatic copper concentration. In addition to investigating the concentration of RONS and AOP, it would be interesting to characterize hepatic copper concentration and lipid peroxidation by measuring isoprostanes. Isoprostanes are a stable product of lipid oxidation that can be measured in blood, urine and tissue.[13] Isoprostanes could have a future role in monitoring on farm oxidative stress through client submitted samples.

In chapter 5, Q5 had significantly more IHC staining for 4HNE and 3NIT than Q1. This finding suggests that super nutritional copper concentration can lead to hepatocellular damage through oxidative stress. However, in our study we only investigated the highest and lowest quintiles. This left the amount of oxidative stress in the middle quintiles a mystery. Future investigations must include the entire range of hepatic copper concentrations in order to examine which concentrations of hepatic copper concentrations are likely to result in oxidative stress. That data could then be used to formulate more accurate interpretation of reference ranges for hepatic copper concentrations in cattle.

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