THESIS

INVESTIGATING HEPATIC COPPER TRAFFICKING IN BEEF CATTLE

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ABSTRACT

INVESTIGATION OF HEPATIC COPPER TRAFFICKING IN BEEF CATTLE

A total of five experiments were conducted to investigate hepatic copper (Cu) trafficking in beef cattle. Two initial experiments were conducted to investigate hepatic Cu trafficking in mice and cattle using proteomics technology. Experiment 1, the objective was to investigate the influence of liver Cu concentrations on the relative abundance of liver Cu homeostatic proteins in beef cattle. Archived liver biopsy samples were selected based on Cu concentrations (n=4 samples 21.7±1.35 mg Cu/kg DM-Cu deficient; and n=4 samples 73.3 ±13.14 mg Cu/kg DM-Cu adequate). Liver samples were obtained from a subset of multiparous beef cows receiving a forage-based diet with no supplemental Cu (basal diet 6.25 mg Cu/kg DM) or 10 mg Cu/kg DM total diet (Cu supplemented as $CuSO_4 \cdot 5H_2O$) for 99 d. Liver proteins were identified using mass spectrometry, normalized, and relative abundance determined using Scaffold software. A total of 895 identical proteins were identified in each sample and relative abundance of each Cu specific homeostatic protein (n= 13) was recorded. Data were analyzed as a randomized complete block design using R software. Copper homeostatic liver proteins identified were: aldehyde dehydrogenase (ALDH2), apolipoprotein A-1(APOA1), betaine homocysteine methyltransferase (BHMT), carbonic anhydrase II (CA2), Cu chaperone for superoxide dismutase (CCS), Cu transport protein (ATOX1), cytochrome c oxidase Cu chaperone (COX17), extracellular superoxide dismutase (SOD3), flavin reductase(BLVRB), glutamate dehydrogenase (GLUD1), glutathione synthetase (GSS), protein disulphide isomerase A3 (PDIA3), and Cu-zinc superoxide dismutase (SOD1). By design, liver Cu concentrations were greater ($P < 0.05$) in Cu adequate

vs. Cu deficient liver samples. Copper deficient liver samples had greater ($P < 0.05$) relative abundance of glutathione synthetase compared to Cu adequate liver samples. The relative abundance of all other Cu homeostatic liver proteins identified were similar ($P > 0.05$) across treatments. These data suggest that deficient and adequate liver Cu concentrations ranging from 16.0 to 109.0 mg Cu/kg DM have minimal impact on the relative abundance of hepatic Cu homeostatic proteins in beef cattle. In experiment 2, the objective was to compare the same Cu homeostatic proteins as described in experiment 1 but across Cu adequate mice (n=8 samples 17.96±0.5 mg Cu/kg DM) and the Cu adequate (n=4 samples 73.3 ±13.14 mg Cu/kg DM) cattle. A total of 670 identical proteins were identified in each mouse liver sample and relative abundance was measured and recorded in the same manner as in experiment 1. Data were analyzed using R and samples were adjusted to a common liver Cu concentration. Results showed that ATOX1, APOA1, BHMT, BLVRB, GLUD1, PDIA3 were differentially expressed ($P< 0.05$) between species. These data suggest relative abundance of proteins involved in hepatic Cu homeostasis may differ across species. Further investigation is needed to determine if liver Cu concentration influences Cu homeostatic protein function. The objectives of the following experiments (experiments 3 and 4) were to investigate: 1) the relative expression of Cu trafficking genes in a subsample of whole bovine liver vs. cultured hepatocytes from a subsample of whole bovine liver and 2) the influence of Cu dose on the relative abundance of Cu trafficking genes in cultured bovine hepatocytes. In experiment 3, liver samples were obtained immediately post-mortem from healthy Angus steers. Total RNA was extracted from a portion of the liver samples, lysed in TRIzol™ and total RNA was isolated. The remaining hepatocytes were isolated and cultured for 1 hour in culture media, then lysed in TRIzol™ and total RNA isolated. In experiment 4, A subset of the above cultured hepatocytes were incubated in media

containing: 0mg/L, 0.10mg/L, 1.0mg/L, 10.0mg/L, and 100mg/L Cu for 1 hour. Cells were collected and lysed in TRIzol™ and total RNA was isolated. For both experiments, quantitative RT-PCR was used to determine the abundance of transcripts for proteins involved in Cu homeostasis in liver tissue and cultured hepatocytes. The identified targets were: ALDH2, APOA1, ATOX1, ATP7A, ATP7B, BHMT, BLVRB, CA2, CCS, COX17, CTR1, ELN, GAPDH, GLUD1, GSS, LOXL1, PDIA3, SOD1, SOD3. B-Actin (ACTB) was selected used as the endogenous control in both experiments. Experiment 3 results indicate a significant ($P < 0.01$) difference between relative expression of mRNA for APOA1, ATOX1, ATP7A, ATP7B, COX17, CTR1, ALDH2, BHMT, BLVRB, CA2, GLUD1, GSS Cu homeostatic genes between a subsample of whole liver and cell culture. Experiment 4 results indicate that all Cu concentrations influences Cu homeostatic genes. Comparing all doses to the control (dose 0), dose 0.1 was only different than 0 for ATP7B. Across all genes measured, dose 1.0 was similar to 0. Dose 10.0 was different than 0 for ALDH2, ATP7A, ATP7B, BLVRB, CA2, CTR1. Dose 100.0 was different than 0 for genes ALDH2, ATOX1, ATP7A, BHMT, COX17, GLUD1, SOD1. Dose 100.0 had the most influence on Cu homeostatic genes. In experiment 5, the objective of the final experiment was to compare gene expression of Cu homeostatic genes from a subset of bovine livers in a coexisting study investigating molybdenum (Mo) supplied in drinking water to primiparous cows. Liver samples were obtained from cows on two treatments within the experiment. Treatments consisted of: 1) no supplemental Cu (-Cu; total diet contained 7.0 mg Cu/kg DM) and 2) 3 mg Cu/kg DM (+Cu; total diet contained 10.0 mg Cu/kg DM). Livers were harvested immediately upon slaughter, rinsed with a phosphate buffered saline solution, diced into small pieces, and placed into RNA later solution. RNA was extracted from all samples in TRIzol™ and total RNA isolated. Quantitative RT-PCR was used to determine the

abundance of transcripts for proteins involved in Cu homeostasis in liver tissue. The identified targets were: ALDH2, APOA1, ATOX1, ATP7A, ATP7B, BHMT, BLVRB, CA2, CCS, COX17, CTR1, ELN, GAPDH, GLUD1, GSS, LOXL1, PDIA3, SOD1, SOD3. Relative abundance of APOA1, ATOX1, ATP7B, BLVRB, CTR1, GLUD1, LOXL1, and SOD3 were greater ($P < 0.05$) in cows receiving supplemental Cu when compared to cattle not receiving supplemental Cu. Although protein expression from experiment 1 were not different in cattle receiving different Cu treatments, gene expression is different for certain Cu homeostatic genes. These results indicate a possible homeostatic difference between cattle supplemented Cu. Further investigation is warranted to determine if a difference in relative gene expression is associated with a difference in protein function.

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CHAPTER 1 − REVIEW OF LITERATURE

INTRODUCTION

Copper (Cu), the twenty-ninth element on the periodic table, is a transition metal commonly found in the divalent (most stable) form. The atomic symbol for copper is Cu and each mole of Cu weighs 63.55 g. Historical records indicate that Cu was used for medicinal purposes dating as early as 400 B.C.E. (Davis and Mertz, 1987). Copper was first identified in plant and animal tissues in the mid 1920's. However, the necessity of Cu in biological systems was not identified until 1928, when the need for Cu in the synthesis of hemoglobin was suggested (Hart et al., 1928). Since that discovery, the role of Cu in critical metabolic functions in both plants and animals has been evident (Underwood and Suttle, 1999).

Copper is found in many essential enzymes important for bone development, reproduction, and other metabolic functions (Underwood and Suttle, 1999). The predominant storage site for Cu is liver with small concentrations of Cu in brain, kidney, and spleen. Distribution of Cu within an animal varies with age, sex, Cu status, and species (Davis and Mertz, 1987). Since the discovery of the importance of Cu in biological functions in mammals, research has focused on investigating functions of Cu requiring enzymes, transmembrane transporters, and intracellular transporters of Cu with minimal focus on species and organ specific Cu related functions.

The majority of data available for feeding Cu to livestock focuses on clinical signs of deficiency or toxicity. Although there has been much progress in our understanding of necessity of Cu, there hasn’t been much translation into practice for the agricultural community (Clarkson et al., 2019). This specific focus does not contain explanation of what is taking place on a

cellular level. Most of the basic molecular research in this area has used rat, mice and human models and primarily focus on understanding human related Cu diseases such as Wilson's and Menke's disease. However, little research has been conducted to determine if applying information learned from murine models regarding Cu homeostasis is appropriate for Cu homeostasis in ruminants. The hypothesis of this thesis is that Cu metabolism in ruminants is different from non-ruminant species. Therefore, the overall objective of this thesis was to investigate hepatic Cu homeostasis in ruminants and compare mechanisms of Cu metabolism identified through technologies such as proteomics, genomics, and cell culture to assist in our understanding of hepatic Cu homeostasis.

COPPER ABSORPTION IN RUMINANTS

Absorption of Cu begins in the small intestine. For Cu to be absorbed, it must be reduced to a reactive state. As described by Spears et al. (2011): Copper enters the enterocyte through the transmembrane transporter copper transporter 1 (CTR1), or a small percentage of Cu enters through divalent metal transporter 1 (DMT1). Once inside the cell Cu: synthesizes cytochrome c oxidase (CCO) in the mitochondria via cyclo-oxygenase 17 (COX17); binds to copper chaperone protein (CCS) to synthesize super oxide dismutase (SOD1); travels via antioxidant 1 (ATOX1), then to ATPase Cu transporting alpha (ATP7A) to the trans-golgi network (TGN) where it binds to metallothionein (MT) and is stored in the lysosome (Spears et al., 2011). Absorption of Cu is regulated by nutritional status as well as the chemical form of the mineral (Davis and Mertz, 1987). It is known that the Cu absorbed in the small intestine can then be transported to other tissues via albumin and other amino acid transporters (Davis and Mertz, 1987).

Minerals exist in biological systems, either in the ionic form or as soluble complexes bound to organic ligands (Thompson and Fowler, 1990). Absorption of minerals is therefore

dependent on solubility. Failure to become solubilized in the intestinal lumen can prevent intestinal absorption (Spears et al., 2011). The pathways of absorption in ruminant species may be similar to those identified in murine models. Copper concentration in diet and water, as well as age, and species can influence Cu absorption (Davis and Mertz, 1987). The efficiency of absorption of many trace minerals is greater in nonruminants than in ruminants (Spears, 2003). As a result, Cu storage is greater in the liver of ruminants (Han et al., 2012). These differences are due to the modifications that occur in the rumen that can have an impact on bioavailability (Spears, 2003). In the ruminant animal, Cu absorption takes place predominantly in the small intestine. Figure 1 describes Cu absorption from an enterocyte and trafficking to the hepatocyte (Figure 1; adapted from Spears et al., 2011). Absorption of Cu from the lumen of the small intestine begins with CTR1. It has been demonstrated that Simmentals have less intestinal CTR1 which suggests reduced ability to absorb Cu from the diet (Fry et al., 2013). This breed specific physiological adaptation indicates possible differences among breeds and needs to be taken into consideration when investigating absorption in bovine species.

TRANSPORT, DELIVERY, AND CELLULAR METABOLISM OF COPPER

After being absorbed from the intestine, Cu is transported to the liver and kidney (Weiss and Linder, 1985). The liver plays a substantial role in Cu metabolism, and it is by this organ that the Cu status of an animal is measured (Davis and Mertz, 1987). The mechanism for Cu transport to tissues is not fully understood in ruminants. The liver regulates circulating Cu levels by absorbing and breaking down the protein ceruloplasmin (Cp) (Harris, 2000). Ceruloplasmin and albumin were previously accepted as Cu transporters, but there was evidence of an additional transport protein in blood plasma (Weiss and Linder, 1985). Using radioisotopes in rats, a new Cu transport protein was identified in the plasma. This Cu carrier was named transcuprein and had not been previously identified (Weiss and Linder, 1985). Transcuprein transports both Cu

and Zn in the blood; the amount of transcuprein depends on Cu and iron (Fe) availability (Liu et al., 2007).

Bile, Cp, and storage were the accepted "pools" of Cu that existed in the liver (Bremner, 1987). In addition to transcuprein, MT is involved in Cu metabolism. Metallothionein is thought to be a storage protein due to its ability to bind to Cu in the liver but its exact mechanism in hepatic metabolism is unclear (Bremner, 1987). The proposed theory is that MT bound to Cu is most likely a response to increased concentrations of Cu in the cell and serves as a detoxification aid (Bremner, 1987). It has been demonstrated that once Cu enters the hepatocyte it has one of four fates within the cell. Copper can join the MT pool for storage, be shuttled to the mitochondria via COX17 to be incorporated with CCO, bind to CCS to synthesize SOD1, or are transported to the TGN via ATOX1 to then leave the cell for absorption via Cp or for excretion via bile (Shim and Harris, 2003). Copper chaperone protein, SOD1, and ATOX1 are categorized as low-molecular-weight proteins called metallochaperones (Roberts and Sarkar, 2008).

EXCRETION OF COPPER

The primary excretion of Cu from the body is through biliary secretion into the intestinal lumen which then exits the body via the feces. Among different species, ruminant animals tend to have lower capacity for excretion and higher capacity for binding of Cu in the liver (Davis and Mertz, 1987). In addition to fecal excretion Cu can be expelled via the urine. However, urinary excretion of Cu is minor, and unaffected by Cu intake across most species (Suttle, 2010).

COPPER DEFICIENCY AND TOXICITY

Supplementation of Cu to beef cattle often exceeds the recommended values provided by the nutritional research council (NRC). The current dietary Cu recommendation for growing and finishing beef cattle and gestating and early lactation beef cattle is 10mg Cu/kg DM with the maximum tolerance of 40 mg Cu/kg DM (NASEM, 2016). This broad range between

requirement and signs of toxicity is a potential reason for over supplementation of this mineral. To evaluate effect of Cu in the diet, liver Cu concentration is used as indicator for deficiency and toxicity (Davis and Mertz, 1987). Whole blood levels of Cu are reported to be similar to reported serum levels, but serum levels are reported as a better indicator of Cu status in cattle (Puls, 1994). Copper is considered deficient when the serum concentration of Cu are within the range of 0.2-1.00ppm wet weight and considered adequate when serum concentration are within the range of 0.32-1.20ppm wet weight. Copper is considered deficient in ruminants when hepatic copper concentrations are between 0.5-10.0ppm wet weight and adequate levels when concentrations are 25-100ppm wet weight (Puls, 1994).

DIETARY COPPER ANTAGONISTS

Trace mineral interactions were first described as elements with chemical and physical properties that are alike will act antagonistically to each other in a biological system (Hill and Matrone, 1970). The results of experiments performed by Hill and Matrone (1970) confirmed that hypothesis and went on to describe that when ions are similar, they interfere physiologically through competition of uptake by tissues and organelles. Digestion of essential trace minerals in ruminants is different compared to digestion in non-ruminant species. One of these differences is the mineral-mineral interactions within the rumen which is a highly reducing environment.

Increased dietary Fe can result in impaired digestive absorption of Cu; however, this does not occur in pre-ruminant calves. This indicates that a fully developed rumen is required for Fe to down regulate Cu absorption (Spears, 2003). Additionally, the presence of thiomolybdates (TM) reduce Cu absorption in ruminant animals (Spears, 2003). Thiomolybdates are formed when high concentrations of sulfur (S) and molybdenum (Mo) are consumed by ruminants. Furthermore, research conducted by Han et al. (2012) involved investigating the relationship of liver tissue and pulmonary artery Cu concentrations and genes involved in Cu homeostasis (Han

et al., 2012). The results indicated that several genes involved in Cu homeostasis have different modes of regulation across species (Han et al., 2012).

Copper, Molybdenum, and Sulfur

A delicate balance exists in the ruminant diet between Cu, Mo, and S. This needs to be considered when formulating diets to avoid the occurrence of deficiency and/or toxicity. Interest of these interactions was piqued by the fact that the observed interaction in ruminants was vastly different than mineral interactions in non-ruminants (Suttle, 1980). Early data by Dick and Bull (1945) suggested that Mo reduces the amount of Cu stored in the liver of ruminant animals. The experiment that led to this conclusion analyzed liver Cu concentrations from cows that had been given daily doses of ammonium molybdate; approximately 904 g over a three-year period. Upon slaughter, liver Cu concentrations were indicative of a Cu deficiency (< 40 mg Cu/kg) and adequate in Mo (3 mg/kg) (Dick and Bull, 1945). Additionally, Dick and Bull (1945) conducted a similar experiment using sheep and concluded that supplemental Mo reduces Cu stored in the liver. These results led to the conclusion that an increase in dietary Mo may induce a Cu deficiency in cattle and sheep. On the other hand, sheep grazing pastures that are low in Mo and normal in Cu may have high Cu status (Dick and Bull, 1945). Research continued over the years and supported the original conclusion of mineral interactions by Hill and Matrone (1970) but the specific site of the interaction between trace minerals was rarely described (Bremner and Beattie, 1995).

There are important differences between ruminants and non-ruminants due to the presence of rumen bacteria and protozoa and their role in dietary Mo and S metabolism in the rumen (Mason, 1978). Due to the presence of microorganisms in the rumen, ruminant animals have the ability to utilize inorganic S to synthesize S-containing amino acids (Kahlon et al.,

1975). This process is unique to ruminant animals, where non-ruminant animals require S in organic form (Kandylis, 1984). Additionally, when S is consumed as either sulfate (SO_4^{2-}) or via an S-containing amino acid, it is converted to sulfide (S^{2-}) in the rumen (Ward, 1978). Rumen microbes use sulfide as a source of S for bacterial synthesis of S-containing amino acids; however, sulfide readily combines with Mo (if present) to form a TM (MoS_4^{-2}) which binds Cu and forms $CuMoS_4$ (Suttle, 1991). When Cu is bound to either S^{2-} or molybdate (MoO_4^{2-}), it is almost completely unabsorbed by ruminants (Huisingh and Matrone, 1976). It is not clear if the Cu-Mo-S complex can be absorbed; however, this complex would be completely unavailable for metabolism by the animal if absorbed (Ward, 1978). Mills and others (1977) evaluated the effects of increasing dietary Mo concentration at either low or high dietary concentrations of S and increasing dietary S concentration at either low or high dietary concentrations of Mo. The authors reported reductions in liver Cu retention when Mo was increased when S concentrations were high and low, and when S was increased at while Mo was at a high concentration; however, liver Cu retention was not affected when dietary S concentration was increased at the same time Mo concentration was low.

There are four documented TM types: mono-, di-, tri- and tetra-thiomolybdate. The TM type is dependent on the S:Mo ratio present in the rumen. At a low S:Mo ratio, monothiomolybdate (TM_1), dithiomolybdate (TM_2), and trithiomolybdate (TM_3) are favorably formed. When S:Mo ratios are high for a long period of time, tetrathiomolybdate (TM_4) is formed (Clarke and Laurie, 1980). Review of previous literature written by N.F. Suttle suggests a delicate balance between Cu, S, and Mo. Molybdenum lowers availability of Cu especially when S is present.

Copper, Mo, and S interactions, and the formation of TM in ruminant species indicate that Mo in the form of MoO_4^{2-} is converted to TM due in part to the S in the form of S^{2-} in the rumen environment (Suttle, 1980). Most of the experimental observations suggest that TM reduces Cu availability; however, these are based on sheep models. Data on TM formation using cattle models is limited (Suttle, 1980). When TM_3 and TM_4 are absorbed into the blood, they can combine with plasma, it reacts with albumin and forms a Cu-TM-albumin complex which is very strong and causes restriction for Cp synthesis from absorbed Cu (Suttle, 1991). It has been suggested that TM formation does not result in Cu deficiency but rather leads to TM toxicity (Gould and Kendall, 2011). Further investigation is needed to determine if this is the case. The Cu x Mo x S interaction has the ability to shift ruminant animals from deficiency to toxicity, this is unlike any other mineral interaction (Suttle, 1991). Interest in mineral-mineral interactions continued when there was a loss of production as a result of Cu deficiency in grazing animals (Suttle, 1980).

Microorganisms within the rumen have been shown to reduce the incidence of Cu toxicity in ruminants. Ivan et al. (1986), conducted an experiment investigating the importance of rumen protozoa in sheep fed high levels of dietary Cu. The absence of rumen ciliate protozoa (fauna-free) caused an outbreak of Cu toxicity in a flock of sheep in 1980 (Ivan et al., 1986). The results indicated that the presence of ciliate protozoa in sheep decreased Cu liver accumulation despite their higher feed intake. This suggests that the fauna-free sheep had higher absorption of Cu and therefore higher accumulation in the liver. Though this experiment demonstrated the importance of rumen ciliate in the prevention of Cu toxicity in sheep, it did not present the effects of Cu solubility in the rumen nor the effect of protozoa on Cu availability (Ivan, 1988). These effects of solubility were demonstrated later in a similar experiment by Ivan (1988) using

a low and high Cu diet fed to both faunated and fauna-free rams. The results demonstrated that on both low and high Cu containing diets, the faunated sheep accumulated less Cu in the liver due to the ability of protozoa to reduce S in the rumen which can interact with Cu and lower availability. Additionally, faunated sheep high or low Cu containing diets had lower cell-free soluble fraction for Cu and lower Cu solubility in the rumen. It is likely that the results of higher levels of Cu in the rumen, blood, and liver were due to the lack of protozoa in the fauna-free sheep. The less protozoa in the rumen result in more stored Cu in the liver which results in more instances of toxicity in sheep.

Copper and Zinc

The concept that the individual form in which an element occurs in a body tissue is indicative of its function and availability was first demonstrated using gel-filtration techniques. Specifically focusing on Cu-Zn interaction, there was evidence confirming protein-binding relationship between these minerals in the ruminant liver (Bremner and Marshall, 1974). Bremner and Marshall (1974) used livers from veal calves involved in other experiments which were fed varying amounts of both Cu and Zn. Calf livers were classified as low-normal Cu (0.5 mg Cu/kg DM) or high Cu (5.5 mg Cu/kg DM). Additionally, sheep livers were obtained and classified into normal Zn (40 mg Zn/kg DM) and deficient Zn (<1.0 mg Zn/kg DM). Zinc concentrations were reported to increase in a Cu-deficient calf liver indicating a possible relationship between homeostatic proteins. Prior research had ignored changes in Zn concentration in the liver, but it seems probable to investigate these changes associated with a metal-binding fraction. The metal binding fraction discussed in the experiment was thought to be similar to MT but had not been identified in terms of function or role (Bremner and Marshall, 1974). Zinc and Cu are of similar chemical and physical properties, (Bremner and Beattie, 1995).

Copper requirements prior to 2000 had not been well- defined for feedlot cattle fed high concentrate diets (Engle and Spears, 2000). Long-term effects of over supplementation of Cu on Zn and Cu status was evaluated and the results indicated that Cu concentrations did not increase during finishing phase contrary to expectations of the experiment; thus, suggesting mechanism to prevent Cu overload in cattle overfed Cu (Engle and Spears, 2000).

Copper and Iron

In addition to the minerals discussed in the previous sections, Campbell et al. (1974) proposed that increased Fe in the diet of ruminants may have a negative, long term effect on Cu status. These effects were shown in their experiment involving yearling cattle dosed with hydrated ferric oxide slurry (210 mg Fe/kg live weight) by mouth on a weekly basis. The estimated intake for the treated animals was 1400 mg Fe/kg diet DM which was about 30 mg Fe/kg live weight additional to what the animal was receiving naturally in the pasture (Campbell et al., 1974). The calves with increased Fe intake had deficient liver Cu concentrations and reduced Cp and amine oxidase activity. The results of this experiment demonstrated that a rapid increase in dietary Fe results in lowering of body Cu concentrations and the function of certain enzymes. The interference of Fe with Cu was hypothesized to be at the cellular or mucosal level or at a stage in the digestive tract (Campbell et al., 1974). Absorption can be impaired when trace minerals interact together through competition for transporters (Spears et al., 2011).

GENETIC DISEASES SPECIFIC TO COPPER

Menke's Disease

Original discovery of Menke's disease (MD) represented evidence of Cu deficiency in humans (Harris, 2000). Menke's disease was initially deemed a recessive disorder which was characterized by strange hair texture and damage to neurons in the brain causing cerebellum weakening (Menkes et al., 1962). The disease results in a mutation of the Cu chaperone ATP7A.

ATPase Cu transporting alpha is normally responsible for the export of absorbed Cu from intestinal cells (Suttle, 2012). The disruption of this pathway results in the inability for Cu to be metabolized by the liver and distributed throughout the body, causing a deficiency.

Wilson's Disease

Wilson's Disease (WD) was originally diagnosed as an autosomal recessive disease in 1912 (Wilson, 1912). Symptoms of WD include hepatic Cu overload and can lead to liver disease in humans (Roberts and Sarkar, 2008). Wilson's Disease is caused from a lack of the Cu chaperone protein ATPase Cu transporting beta (ATP7B). ATPase Cu transporting beta normally incorporates Cu into CP (Suttle, 2012). With the loss of production of ATP7B the Cu does not have the ability to leave the hepatocyte via blood or bile resulting in toxicity of Cu in humans. This is also a possible explanation for Cu sensitivity in certain sheep breeds (Spears et al., 2011).

TECHNOLOGY TO BETTER UNDERSTAND COPPER HOMEOSTASIS

Proteomics and Genomics

The use of proteomics and genomics has allowed for a more in-depth understanding of mammalian metabolism and function of various tissues and cells. With the influence of disease in humans, specifically childhood cirrhosis due to Cu toxicity, research has been conducted using a sheep model to analyze Cu responsive liver proteins (Simpson et al., 2004). The authors discussed the use of North Ronaldsay sheep as a possible model in the future due to their sensitivity to Cu in the environment. They decide to use Cambridge sheep as a model because they are a more Cu tolerant breed (Simpson et al., 2004). The authors suggested that the differences in sheep breeds may be due to metabolism and excretion differences (Simpson et al., 2004). The results of this experiment identified Cu responsive proteins using proteomic techniques and showed that these proteins were differentially expressed in Cu deficient sheep

(Simpson et al., 2004). Aldehyde dehydrogenase (ALDH2) was upregulated in sheep that had high concentrations of Cu in the diet (Simpson et al., 2004). Aldehyde dehydrogenase binds to retinal proteins, forming a retinal bound complex which may minimize the effects of copper-induced oxidative stress (Simpson et al., 2004). Apolipoprotein A-1 (APOA1) was down regulated in Cu-challenged sheep which may indicate disruption of the protein synthesis due to their secretion function (Simpson et al., 2004). Betaine homocysteine methyltransferase (BHMT) is a liver enzyme shown to be upregulated in Cu-challenged sheep (Simpson et al., 2004). Betaine homocysteine methyltransferase is a Cu dependent enzyme that synthesizes methionine (Simpson et al., 2004). Carbonic anhydrase II (CA2) was significantly upregulated in Cu challenged sheep; CA2 is an isoform of carbonic anhydrase III which is known in hepatocytes to be glutathionylated during oxidative stress (Simpson et al., 2004). Thus, the discovery of this upregulated protein is consistent with an oxidative stress response in Cu-challenged sheep (Simpson et al., 2004).

Data suggests that ATOX1 is required for full activity of superoxide dismutase enzymes (Jeney et al., 2005). Cytochrome c oxidase Cu chaperone is important for delivery of Cu to the mitochondria within enterocytes and liver cells during Cu homeostasis to then synthesize CCO;(Prohaska, 2008). Flavin reductase (BLVRB) in addition to other enzymes previously listed also protects cells from oxidative damage (Simpson et al., 2004). Flavin reductase was up-regulated in copper-challenged sheep. Glutamate dehydrogenase (GLUD1) was up-regulated in liver containing high levels of copper (Simpson et al., 2004). Glutamate dehydrogenase is an enzyme involved in the production of NADPH (Simpson et al., 2004). Glutathione synthetase (GSS) was elevated in Cu-challenged livers; deficiency of GSS can lead to inability to resist oxidative stress (Simpson et al., 2004). Therefore, it is unsurprising that in an elevated Cu state

that this enzyme would be upregulated to protect against free radicals. Protein disulphide isomerase A3 (PDIA3) was upregulated in the Cu challenged sheep, this is likely to be in response to oxidative challenged presented by Cu (Simpson et al., 2004). Protein disulphide isomerase A3 is found in the endoplasmic reticulum and plays a role in protein folding (Simpson et al., 2004). Superoxide dismutase [Cu-Zn] was not investigated in the experiment by Simpson et al. (2004) but was found to have reduced relative abundance of mRNA in Cu deficient livers, which indicated it's Cu dependence for expression (Suazo et al., 2008). Copper chaperone protein is important for shuttling Cu to SOD1 in the cytosol of enterocytes as well as hepatocytes during Cu homeostasis (Spears et al., 2011). Copper chaperones are essential to sequester and deliver Cu to their targets (Wong et al., 2000). Extracellular superoxide dismutase (SOD3) is a Cu dependent isomer of SOD1 and activity of SOD3 has been shown to have a dependent relationship with its Cu content (Jeney et al., 2005). Copper Transporter 1(CTR1) is the primary transmembrane protein responsible for importing dietary Cu into the liver (Prohaska, 2008). ATPase Cu transporting alpha is a transmembrane protein that transfers Cu from the intestine to the liver and ATP7B is a intracellular transporter within liver cells that is involved in metabolizing Cu. Lysyl Oxidase (LOXL1) is a Cu dependent enzyme involved in elastin (ELN) formation (Han et al., 2012). It has been investigated to see if gene and protein expression of Cu chaperones could serve as biomarkers for Cu status in humans with minimal success (Prohaska, 2008). However, data from Han et al. (2012) indicated that genes involved in Cu homeostasis were significantly different across different species (Han et al., 2012). Therefore, although it was unsuccessful to use gene and protein abundance as markers for homeostasis in humans, there is possibility that they can be used as markers in bovine.

Cell culture

The use of cell culture as a means of understanding mineral metabolism in many species has been a successful model. It presents a controlled environment that can be measured and replicated to better understand homeostasis of the body. Majority of published data investigating culturing hepatocytes have used mice, rat, or human models. This is not only due to access of these subjects but also because liver diseases such as WD is of primary interest. Research specific to ruminant mineral metabolism is limited. A key aspect of liver cell culture is enzyme digestion. Digestion of liver tissue via enzyme was first introduced in 1967; enzyme digestion had been used successfully to harvest cells from other tissues but not from liver tissue (Howard et al., 1967). Howard et al. (1976) method used incubation, enzyme solution, and mechanical treatment. Results yielded 75% structural integrity (Howard et al., 1967). This breakthrough in the successful use of enzyme digestion of liver tissue allowed for better understanding of liver metabolism in rats. Shortly after, Berry and Friend (1969) focused on the limitation of Howard et al. (1967) research in that only 5% of the overall tissue is represented with their approach (Berry and Friend, 1969). Berry and Friend developed a technique that involved continuous recirculation of an enzyme solution followed by perfusion. This technique yielded six times the yield of hepatocytes of what Howard et al. (1967) research produced (Berry and Friend, 1969). This method still had its limitations, the perfusion of the liver resulted in swelling and therefore cell death. The improvement of this method continued with the introduction of the two step procedure (Seglen, 1976).

Perfusion techniques were thought to be essential to successfully obtain liver cells; however, when using a large model such as bovine, these techniques are challenging (Spotorno et al., 2006). A non-perfusion technique presented a starting point for a hepatocyte isolation that

is simple to apply (Spotorno et al., 2006). Further investigation is warranted to build on this technique and obtain a greater understanding of Cu homeostasis specific to ruminants. Using this method, we hope to achieve such understanding.

CONCLUSION

Copper homeostasis in ruminants is a biologically relevant area of research. Copper has many essential functions across all species of mammals. Although much progress has been made over time in ruminant trace mineral nutrition, most of the research examining hepatic Cu homeostasis has been conducted in rodent models. It is important therefore to understand bovine specific Cu metabolism to more efficiently supplement Cu to the animal. The research in this thesis attempts to investigate Cu homeostasis using gene and protein abundance of specific Cu homeostatic and Cu responsive genes/proteins found in the literature. If it is determined that the relative abundance of these Cu homeostatic proteins are different with increasing or decreasing levels of Cu concentration, further research will be needed to evaluate if these observed responses dictate function and if these functions differ between ruminant and non-ruminant species.

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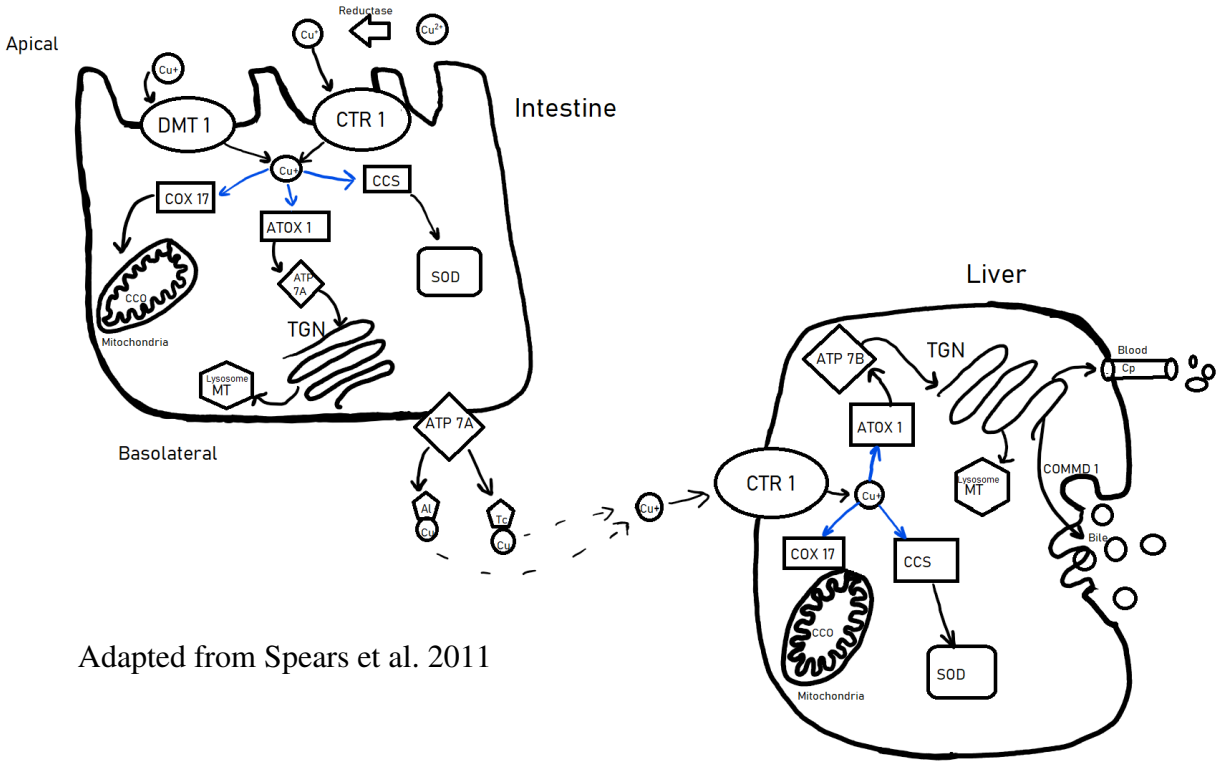

Adapted from Spears et al. 2011

Figure 1.1 Intestinal Cu absorption and secretion from liver

CHAPTER 2 − THE INFLUENCE AND COMPARISON OF LIVER COPPER CONCENTRATION ON COPPER HOMEOSTATIC LIVER PROTEINS IN BEEF CATTLE AND MICE

SUMMARY

Two experiments were conducted to investigate Cu homeostatic proteins and the influence that liver Cu status, and species has on the relative abundance of the identified proteins. Experiment 1, the objective was to investigate the influence of liver Cu concentrations on the relative abundance of liver Cu homeostatic proteins in beef cattle. Archived liver biopsy samples were selected based on Cu concentrations (n=4 samples 21.7±1.35 mg Cu/kg DM-Cu deficient; and n=4 samples 73.3 ±13.14 mg Cu/kg DM-Cu adequate). Liver samples were obtained from a subset of multiparous beef cows receiving a forage-based diet with no supplemental Cu (basal diet 6.25 mg Cu/kg DM) or 10 mg Cu/kg DM total diet (Cu supplemented as $CuSO_4 \cdot 5H_2O$) for 99 d. Liver proteins were identified using mass spectrometry, normalized, and relative abundance determined using Scaffold software. A total of 895 identical proteins were identified in each sample and relative abundance of each Cu specific homeostatic protein (n= 13) was recorded. Data were analyzed as a randomized complete block design using R software. Copper homeostatic liver proteins identified were: aldehyde dehydrogenase (ALDH2), apolipoprotein A-1(APOA1), betaine homocysteine methyltransferase (BHMT), carbonic anhydrase II (CA2), Cu chaperone for superoxide dismutase (CCS), Cu transport protein (ATOX1), cytochrome c oxidase Cu chaperone (COX17), extracellular superoxide dismutase (SOD3), flavin reductase (BLVRB), glutamate dehydrogenase (GLUD1), glutathione synthetase (GSS), protein disulphide isomerase A3 (PDIA3), and Cu-zinc superoxide dismutase (SOD1). By design, liver Cu concentrations were greater ($P < 0.05$) in Cu adequate vs. Cu deficient liver samples. Copper deficient liver samples had greater ($P < 0.05$) relative abundance of glutathione synthetase compared to Cu adequate liver samples. The relative abundance of all

other Cu homeostatic liver proteins identified were similar ($P > 0.05$) across treatments. These data suggest that deficient and adequate liver Cu concentrations ranging from 16.0 to 109.0 mg Cu/kg DM have minimal impact on the relative abundance of hepatic Cu homeostatic proteins in beef cattle. In experiment 2, the objective was to compare the same Cu homeostatic proteins as described in experiment 1 but across Cu adequate mice (n=8 samples 17.96±0.5 mg Cu/kg DM) and the Cu adequate (n=4 samples 73.3 ±13.14 mg Cu/kg DM) cattle. A total of 670 identical proteins were identified in each mouse liver sample and relative abundance was measured and recorded in the same manner as in experiment 1. Data were analyzed using R and samples were adjusted to a common liver Cu concentration using a covariate. Results showed that ATOX1, APOA1, BHMT, BLVRB, GLUD1, PDIA3 were differentially expressed ($P < 0.05$) between species. These data suggest relative abundance of proteins involved in hepatic Cu homeostasis may differ across species. Further investigation is needed to determine if liver Cu concentration influences Cu homeostatic protein function.

INTRODUCTION

Copper is an essential nutrient required for numerous functions including iron metabolism, blood formation, bone formation, lipid metabolism, and fat deposition, cellular respiration, hair/wool pigmentation, immune system health, and central nervous system function (Davis and Mertz, 1987). Optimizing these functions are particularly important to beef producers. Most data available for feeding Cu to cattle focuses on clinical signs of deficiency or toxicity. This specific focus does not contain any physiological explanation of why these signs appear or what is taking place on a cellular level. Published data indicates that species, age, and composition of the diet determine Cu content of the liver with no reference to function (Underwood, 1971). The majority of basic molecular research in this area has used rat, mice and

human models and primarily focuses on understanding human related Cu diseases such as Wilson's and Menke's disease. However, little research has been conducted to determine if applying information learned from murine models regarding Cu homeostasis is appropriate to apply to Cu homeostasis in ruminants. Therefore, the objective of this experiment was to compare hepatic Cu responsive proteins across murine and bovine species to assess if the protein profiles of these species were similar. Additionally, the objective of this experiment was to compare Cu responsive protein profiles within bovine livers to determine if liver Cu concentration influences the relative abundance of Cu responsive proteins. Our hypothesis was that murine and bovine liver Cu homeostatic proteins would differ by species and that liver Cu concentration would alter Cu responsive proteins associated with Cu homeostasis.

MATERIALS AND METHODS

Experiment 1 – Bovine liver tissue was obtained from residual biopsy samples from an unrelated experiment investigating the influence of Cu supplementation on reproductive performance of multiparous beef cows. Cows received a forage-based diet with no supplemental Cu (basal diet 6.25 mg Cu/kg DM) or 10 mg Cu/kg DM total diet (Cu supplemented as $CuSO_4 \cdot 5H_2O$) for 99 d. Biopsies were collected and immediately rinsed with a phosphate buffered saline (PBS) solution. Samples were then placed in a 1.5mL cryovial and flash frozen in liquid nitrogen. Samples were then transported to back to the laboratory and stored at -80°C.

Experiment 2 – Mice liver samples were obtained from the Painter Center at Colorado State University. Mice were euthanized and whole livers were immediately rinsed with PBS, collected in 1.5mL cryovials, and flash frozen in liquid nitrogen. Samples were then transported back to the laboratory and stored at -80°C. Proteomics analysis was conducted at the proteomics and metabolomics facility at Colorado State University.

Sample Preparation – Protein Extraction & Quantification: After thawing, liver tissue was rinsed with PBS and manually cut into thin strips. Approximately 200mg of tissue sample was weighed and combined with an equal volume (200mg) of 0.2mm zirconium oxide beads. 600µl of homogenization buffer was then added to each sample and samples were then homogenized in a Bullet Blender 5 Storm by Next Advance, Inc. (2113 NY-7 Troy, NY 12180) at speed 8 for 3 minutes followed by speed 9 for 1 minute. Homogenates were then transferred to 1.5mL microcentrifuge tubes and subjected to cup horn sonication (amplitude 70, 10s pulse followed by 20s rest; 8 minutes total sonication). 200µl of homogenate was then aliquoted and combined with 1mL ice cold 3:2 Methyl tert-butyl ether:70% Methanol. Samples were agitated for 30 minutes followed by 3 hours at -80˚C. Cell debris and precipitated proteins were then centrifuged 17,000 x *g* at 4˚C for 10 minutes. Pellets were washed with 300µl 3:2 Methyl tert-butyl ether:70% Methanol (MeOH) and centrifuged again. Pellets were then subjected to two more washes (100%MTBE and 100% acetone) and centrifugations followed by air drying under foil. Pellets were reconstituted in 200µl 2M urea and bath sonicated for 5 minutes. Cell debris was removed via centrifugation at 4,000 x *g* for 2 minutes. An aliquot of the supernatant was diluted 1:5 and subjected to a Pierce™ BCA Protein Assay following manufacturer's instructions. Mice and cattle liver samples were digested via trypsin digestion. Approximately 50 µg total protein was aliquoted from each sample. Trypsin was added to each tube and incubated for 3 h. Briefly, protein was resolubilized in 8M urea, 0.2% ProteaseMAX™ surfactant trypsin enhancer by Promega Corporation (2800 Woods Hollow Road Madison, WI 53711). Samples were reduced and alkylated with 5mM dithiothreitol and 5mM iodoacetamide. Trypsin (Pierce MS-Grade, Thermo Scientific) was added at an enzyme to substrate ratio of 1:50 and incubated at 37°C for 3h. Trypsin was deactivated with the addition of 5% trifluoroacetic acid and desalted using

Pierce C18 spin columns by Thermo Scientific (3747 N. Meridian Road Rockford IL 61101) using manufacturer's instructions. Peptide eluate was dried in a vacuum evaporator and resuspended in 5% acetonitrile/0.1% formic acid. Once resolubilized, absorbance at 205nm was measured on a NanoDrop by Thermo Scientific (3747 N. Meridian Road Rockford IL 61101) and total peptide concentration was subsequently calculated using an extinction coefficient of 31.

Mass Spectrometry Analysis – A total of 1µg of peptides were purified and concentrated using an on-line enrichment column (Waters Symmetry Trap C18 100Å, 5µm, 180 µm ID x 20mm column). Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Presentation of Results - Copper homeostatic proteins were identified through basic search in Scaffold software from Proteome Software, Inc (1340 SW Bertha Blvd, Suite 10 Portland, Oregon 97219). Quantification of proteins for statistical purposes were reported in relative abundance. Total mineral analysis was completed using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES). Mineral analysis was completed on cow and mice liver tissue to define Cu concentrations (Braselton et al., 1997). Mice liver Cu concentration was averaged across samples (n=8 samples 17.9±1.42 mg Cu/kg DM), and cattle were then blocked by liver Cu concentration into adequate and deficient blocks; (n=4 samples 21.7±1.35 mg Cu/kg DM-Cu deficient; and n=4 samples 73.3 ±13.14 mg Cu/kg DM-Cu adequate).

STATISTICAL ANALYSIS

R software was used for all analysis of data. Data were analyzed as a randomized complete block design. The experimental unit was liver (N=16) within experiment. Treatment was included in the model as a fixed classification effect. Within bovine species, samples were blocked by liver Cu concentration (n=4 samples 21.7±1.35 mg Cu/kg DM-Cu deficient; and n=4 samples 73.3 ±13.14 mg Cu/kg DM-Cu adequate). Data was analyzed using the Type III ANOVA procedure in the 'car' package (Fox and Weisberg, 2011), a pairwise comparison was analyzed using species by protein using the emmeans procedure in the 'emmeans' package (Lenth et al., 2019), of R© (R Core Team, 2018). Liver Cu concentration were analyzed using mineral values as a covariate for experiment 2. The effect of treatment was determined significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

Experiment 1: Table 2.1 summarizes the results of experiment 1. The items being referenced in table 1 are the identified Cu homeostatic liver proteins and the liver Cu concentration obtained during mineral analysis. Table 2.1 compares the difference in relative abundance of the Cu homeostatic proteins between Cu adequate and Cu deficient liver samples from beef cattle. By design, liver Cu concentrations were greater ($P < 0.05$) in Cu adequate vs. Cu deficient liver samples. Copper deficient liver samples had greater ($P < 0.05$) relative abundance of glutathione synthetase compared to Cu adequate liver samples. The relative abundance of all other Cu homeostatic liver proteins identified were similar ($P > 0.05$) across Cu concentrations. We reject our null hypothesis that liver Cu concentration would alter Cu responsive proteins associated with Cu homeostasis.

Experiment 2: Tables 2.2 and 2.3 summarize the results of experiment 2. The items being referenced in table 2.2 and 2.3 are the identified Cu homeostatic liver proteins and the liver Cu concentration obtained during mineral analysis. Table 2.2 compares the difference in relative abundance of the Cu homeostatic proteins between Cu adequate bovine liver and Cu adequate murine liver samples. SOD3, ALDH2, and CA2 were the only proteins not ($P > 0.05$) different in bovine and murine liver samples. All other proteins, ATOX1, SOD1, APOA1, BHMT, BLVRB, GLUD1, GSS, and PDIA3 were different ($P < 0.05$), indicating a possible difference in homeostatic mechanisms between species. Due to the extreme difference in Cu liver concentrations between species, liver Cu concentrations were used as a covariate in the statistical model to adjust all samples to a common liver Cu concentration. The relative abundance of ATOX1, APOA1, BHMT, BLVRB, GLUD1, and PDIA3 was different ($P < 0.05$) across species. We fail to reject our null hypothesis that murine and bovine Cu homeostatic liver proteins differ due to species.

CONCLUSION

These data agree with previously published data indicating that liver Cu concentration is higher in steers receiving supplemental Cu in the diet (Engle and Spears, 2000). These data also agree with data that indicates that Cu responsive liver proteins are differentially expressed between control and Cu treated animals (Simpson et al., 2004). These data suggest that deficient and adequate liver Cu concentrations ranging from 16.0 to 109.0 mg Cu/kg DM have minimal impact on the relative abundance of Cu homeostatic proteins in beef cattle and indicate a difference in homeostatic mechanisms of Cu between mice and cattle. Further investigation is needed to determine if liver Cu concentration influences Cu homeostatic protein function.

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Table 2.1 The effects of liver copper (Cu) concentration on relative abundance of Cu homeostatic liver proteins in beef cattle.

Item	Cu Adequate	Cu Deficient	SEM	P<
Liver Cu Concentrations, mg/kg DM	73.30	21.68	11.28	0.015
Aldehyde dehydrogenase (ALDH2)	40.19	54.15	3.52	0.05
Apolipoprotein A-1 (APOA1)	55.59	56.45	2.89	0.90
Betaine homocysteine methyltransferase (BHMT)	95.06	84.61	4.35	0.29
Carbonic anhydrase II (CA2)	8.26	3.87	1.42	0.16
Cu chaperone for superoxide dismutase (CCS)	2.19	3.58	0.59	0.31
Cytochrome c oxidase Cu chaperone (COX17)	1.09	0.86	0.29	0.73
Extracellular superoxide dismutase (SOD3)	3.22	6.55	1.24	0.24
Flavin reductase (BLVRB)	22.97	23.23	1.04	0.92
Glutamate dehydrogenase (GLUD1)	53.50	35.28	6.20	0.18
Glutathione synthetase (GSS)	6.61	8.59	0.49	0.04
Protein disulphide isomerase A3 (PDIA3)	35.11	23.75	2.93	0.06
Superoxide Dismutase [Cu-Zn] (SOD1)	71.29	103.64	9.59	0.12

Table 2.1. The effects of liver Cu concentration on relative abundance of Cu homeostatic liver proteins in beef cattle and mice.

Item	Cu Adequate Bovine	Cu Adequate Murine	SEM	P<
Liver Cu Concentrations, mg/kg DM	73.30	17.96	5.10	0.0003
Aldehyde dehydrogenase (ALDH2)	40.19	31.06	2.55	0.10
Antioxidant 1 copper chaperone (ATOX1)	9.48	14.14	0.66	0.005
Apolipoprotein A-1 (APOA1)	55.59	42.13	2.36	0.017
Betaine homocysteine methyltransferase (BHMT)	95.06	232.72	10.66	0.00007
Carbonic anhydrase II (CA2)	8.26	9.37	1.24	0.66
Extracellular superoxide dismutase (SOD3)	4.30	4.42	0.83	0.94
Flavin reductase (BLVRB)	22.97	13.74	1.15	0.002
Glutamate dehydrogenase (GLUD1)	53.50	21.74	3.05	0.0005
Glutathione synthetase (GSS)	6.61	3.63	0.71	0.06
Protein disulphide isomerase A3 (PDIA3)	35.11	21.71	2.17	0.01
Superoxide Dismutase [Cu-Zn] (SOD1)	71.29	39.61	4.17	0.003

Table 2.2. The effects of species on relative abundance of Cu homeostatic liver proteins in beef cattle and mice adjusted to a common liver Cu concentration

Item	Cu adjusted Bovine	Cu adjusted Murine	SEM	P<
Aldehyde dehydrogenase (ALDH2)	41.73	30.29	5.93	0.31
Antioxidant 1 Copper Chaperone (ATOX1)	8.39	14.68	2.33	0.04
Apolipoprotein A-1 (APOA1)	55.59	42.13	2.36	0.017
Betaine homocysteine methyltransferase (BHMT)	95.28	232.6	44.60	0.013
Carbonic anhydrase II (CA2)	5.05	10.97	2.71	0.25
Extracellular superoxide dismutase (SOD3)	1.37	5.52	1.53	0.163
Flavin reductase (BLVRB)	27.11	11.67	4.19	0.005
Glutamate dehydrogenase (GLUD1)	59.81	18.13	11.95	0.008
Glutathione synthetase (GSS)	5.24	4.31	1.59	0.75
Protein disulphide isomerase A3 (PDIA3)	43.57	17.48	7.66	0.008
Superoxide Dismutase [Cu-Zn] (SOD1)	50.62	49.94	7.08	0.96

CHAPTER 3 − THE IMPACT OF CELL CULTURE AND COPPER DOSE ON COPPER TRAFFICKING GENES IN BOVINE LIVER

SUMMARY

The objectives of the following experiments (experiments 3 and 4) were to investigate: 1) the relative expression of Cu trafficking genes in a subsample of whole bovine liver vs. cultured hepatocytes from a subsample of whole bovine liver and 2) the influence of Cu dose on the relative abundance of Cu trafficking genes in cultured bovine hepatocytes. Experiment 3: liver samples were obtained immediately post-mortem from healthy Angus steers. Total RNA was extracted from a portion of the liver samples, lysed in TRIzol™ and total RNA was isolated. The remaining hepatocytes were isolated and cultured for 1 hour in culture media, then lysed in TRIzol™ and total RNA isolated. Experiment 4: A subset of the above cultured hepatocytes were incubated in media containing: 0mg/L, 0.10mg/L, 1.0mg/L, 10.0mg/L, and 100mg/L Cu for 1 hour. Cells were collected and lysed in TRIzol™ and total RNA was isolated. For both experiments, quantitative RT-PCR was used to determine the abundance of transcripts for proteins involved in Cu homeostasis in liver tissue and cultured hepatocytes. The identified targets were: ALDH2, APOA1, ATOX1, ATP7A, ATP7B, BHMT, BLVRB, CA2, CCS, COX17, CTR1, ELN, GAPDH, GLUD1, GSS, LOXL1, PDIA3, SOD1, SOD3. B-Actin (ACTB) was selected used as the endogenous control in both experiments. Experiment 3 results indicate a significant ($P < 0.01$) difference between relative expression of mRNA for APOA1, ATOX1, ATP7A, ATP7B, COX17, CTR1, ALDH2, BHMT, BLVRB, CA2, GLUD1, GSS Cu homeostatic genes between a subsample of whole liver and cell culture. Experiment 4 results indicate that all Cu concentrations influences Cu homeostatic genes. Comparing all doses to the control (dose 0), dose 0.1 was only different for ATP7B. Across all genes measured, dose 1.0 was similar to 0. Dose 10.0 was different for ALDH2, ATP7A, ATP7B, BLVRB, CA2, CTR1.

Dose 100.0 was different than 0 for genes ALDH2, ATOX1, ATP7A, BHMT, COX17, GLUD1, SOD1. Dose 100.0 had the most influence on Cu homeostatic genes. The results of these experiments indicate a difference between in vitro and in vivo methods as well as a Cu dose response on relative gene expression of Cu homeostatic genes.

INTRODUCTION

The use of cell culture has been a successful model to understand mineral metabolism in many species. It presents a controlled environment that can be precisely manipulated and replicated to better understand tissue specific handling of minerals. The majority of research investigating liver cell culture has utilized liver tissue from mice and rats. Research specific to ruminant hepatic mineral metabolism is limited. Perfusion techniques were thought to be essential to successfully obtain hepatocytes; however, when collecting tissue from large organs such as bovine liver, it is difficult to perfuse the tissue appropriately (Spotorno et al., 2006). In 2006, a non-perfusion technique was introduced by Spotorno et al. (2006). With the published work by Spotorno et al. (2006), a comprehensive and simple protocol was generated as a starting point for cell culture of bovine hepatocytes to be used in comparative studies. Therefore, the objectives of these experiments were to investigate: 1) the relative expression of Cu trafficking genes in whole bovine liver and cultured cells from whole bovine liver and 2) the influence of Cu dose on the relative abundance of Cu trafficking genes in cultured bovine hepatocytes. Our hypothesis was that *in vitro* and *in vivo* studies on bovine liver would be comparable to one another in terms of relative gene abundance. Additionally, our hypothesis was that Cu concentrations ranging from 0-100 mg Cu/L would have an impact on Cu homeostatic gene expression in an *in vitro* environment.

MATERIALS

Reagents and solutions

Phosphate Buffered Saline (PBS; Catalog #10010023), Hank's Balanced Salt Solution (HBSS)-Fisher Scientific (Catalog # 14025076), HEPES 1M-Fisher Scientific (Catalog # 15630080), HBSS-HEPES Solution:10mM HEPES concentration, 45mL HBSS, 10µL HEPES 1M. Collagenase II- 305 U/mg- Fisher Scientific (Catalog #17101015), Collagenase Solution: 1mL of HBSS to 1g Collagenase, Transfer to tube To make 1000X stock solution (100 U/µL) use 3.05mL of HBSS + 1g Collagenase. Collagenase Solution- 250U/mL of HBSS: Digest tissue in 50mL conical tubes so add 100µL of 1000X stock solution per 45mL HBSS-HEPES solution per digestion. Belly Button Shaker- Fisher Scientific (Catalog #15453903), 40µm Cell Strainer-Fisher Scientific (Catalog # 22363547), DMEM- +GlutaMAX, pyruvate- Fisher Scientific (Catalog # 10567022), FBS- Fetal Bovine Serum- Fisher Scientific (Catalog # 10437028), P/S-Penicillin Streptomycin Solution- Fisher Scientific (Catalog # 15140122), Complete Medium: DMEM+GlutaMAX+Pyruvate-500mL, 10% FBS- 50mL, 1% P/S- 5mL. Trypan Blue- Fisher Scientific (Catalog # T10282), Automated Cell Counter-Countless II FL- Fisher Scientific (Catalog # AMQAF1000), 6 well cell culture plates, 1.8cm Cell scrapers- Fisher Scientific (Catalog # 08100241), TRIzol™ - Fisher Scientific (Catalog #15596018). 2 mL 0.8mm Garnet Bead tube- Benchmark Scientific (Catalog# D1033-30G), Bead Bug Microtube Homogenizer-Benchmark Scientific (Catalog # D1030), TURBO DNA-free Kit-Ambion (Catalog # AM1907), NanoDrop™ One Microvolume UV-Vis Spectrophotometer- Fisher Scientific (Catalog # ND-ONE-W).

METHODS

Cell Culture Procedure

Experiment 4: Bovine hepatocytes were cultured using procedures adapted from Spotorno et al. (2006) with modifications. Briefly, samples were weighed in weigh boat and minced prior to placing in PBS. Post mincing with sterile scissors and rinsing with sterile PBS, samples were transferred to 50mL conical tubes containing 45mL of a collagenase solution (250U/mL), placed in incubator at 37°C with 5% CO_2 and constantly agitated (belly button shaker; Fisher Scientific LLC; 4500 Turnberry Drive Hanover Park, IL 60133) on low speed (approx. 25 revolutions per minute) for 18 minutes. Samples were filtered using a 40µm cell strainer (Spotorno et al., 2006).

Portions of caudate lobe of liver from healthy steers were collected from a commercial abattoir using a sterile scalpel blade for each liver sampled. Following collections, each sample was submerged in 70% ethanol for 30 seconds to reduce superficial bacterial contamination. Samples were then placed into a sterile bag, placed on ice, and transported to the laboratory (approximately 30 minutes of travel time from collection to the laboratory). Samples were then aseptically obtained from the geometric center of each liver sample. Approximately 1 to 5 g of each sample was placed into a sterile container, weighed, and minced finely with sterile scissors. Samples were then rinsed 2 times with PBS. Samples were then transferred with sterile forceps to separate 50mL conical tubes containing 45mL of cold (4°C) collagenase solution (250U/mL). Samples were then incubated at 37°C with 5% CO_2 for 18 minutes. Tubes were continuously agitated (belly button shaker; Fisher Scientific LLC; Hanover Park, IL 60133) on low speed (approx. 25 revolutions per minute) during digestion. Post digestion, samples were filtered through sterile 40µm cell strainer into new 50mL conical tubes. Samples were then centrifuged at 50 x *g* for 5min at 4°C. The supernatant was decanted, and the pellet washed with 10mL of

PBS and centrifuged at 150 x g for 2 min and 4°C. This step was repeated twice. The pellet containing the washed hepatocytes was then resuspended in 12mL of Complete Medium. An aliquot (~100µL) of cell-medium solution was removed and Trypan blue exclusion test was performed to estimate cell numbers using an automated cell counter from Fisher Scientific LLC. (4500 Turnberry Drive Hanover Park, IL 60133). Seeding density of cells was 2.46 x 10^5/mL per well. Two mL were transferred to each well in 6 well cell culture plate (approximating ~ 450,000 hepatocytes/well). The six well culture plate was placed in incubator at 37°C with 5% CO_2 for 1hour. After 1 h of incubation, plates were removed, and Cu treatments were applied. Copper treatments were added at a volume of 10µL per well. Copper treatments included: 0.0, 0.1, 1.0, 10.0, 100.0 mg Cu/L. Ten microliters of DI water were added to the wells receiving no Cu. Plates were then incubated for 1 h at 37°C with 5% CO_2. After on hour, media containing hepatocytes was removed and placed in a 10mL conical tube, centrifuged for 2 min at 150 x *g* and the supernatant was removed and placed in Cryovial tubes containing TRIzol™ and vortexed for one minute each. Samples were frozen in -80°C freezer until RNA isolation could be accomplished.

RNA Isolation and cDNA Procedure

Experiment 3: For liver tissue samples, on average 60 mg was added to a 2.0 mL 0.8mm garnet bead tube with 1mL TRIzol reagent. Samples were homogenized with a Bead Bug Microtube Homogenizer from Benchmark Scientific (2600 Main Street Extension, Sayreville, NJ 08872). The DNAse treatment was conducted with a TURBO DNA-free Kit. Post DNA-se treatment, liver samples averaged 4400 ng/µL of RNA. All liver samples were normalized to 500 ng/µL. Post cDNA conversion, liver samples were diluted using equal parts water to cDNA volume and like samples were pooled and aliquoted to minimize freeze thaw cycles.

Experiment 4: For cell culture samples, approximately 450,000 cells were added to a 1.5mL tube with 1mL TRIzol reagent. Samples were vortexed on high for 1 minute. Cell samples from treatment 0, 0.1, 1.0, and 10.0 mg Cu/L averaged 12 ng/µL of RNA. Cell samples from treatment 100 mg Cu/L yielded 60 ng/µL of RNA. Omitted DNA-se treatment for cell samples due to low yield of RNA. Additionally, standard cDNA protocol was modified to account for low RNA yield: In step 1, 9µL of template was used instead of standard 4 µL volume. In step 2, 10mM dNTP was used instead of standard 2mM. Samples were also not diluted with equal parts water and instead, like samples were pooled together to allow for maximum cDNA yield comparable to that from experiment 3.

The following procedures were the same for both experiments: Total RNA was isolated from bovine liver tissue and cultured hepatocytes using TRIzol™ reagent. In brief, the steps of the protocol included: homogenization, phase separation, RNA precipitation, washing, and solubilization (Chomczynski, 1993). Concentrations and purity were estimated with a NanoDrop™ One Microvolume UV-Vis Spectrophotometer from Fisher Scientific (4500 Turnberry Drive Hanover Park, IL 60133). Samples were then converted to cDNA using a standard protocol and combination of random hexamer and oligo-dT primers.

CALCULATIONS

The $2^{-\Delta\Delta C_T}$ method was used to quantify the results from the RT-PCR analysis (Livak and Schmittgen, 2001). For each amplification reaction, QuantStudio™ Design and Analysis Software 1.3.1 (applied biosystems by ThermoScientific) provided a quantified value for time taken for the florescent signal to cross threshold. The value calculated was defined as the cycle threshold (C_T). The 18 genes selected will be referred to in the calculations as ‘Gene of Interest’, ACTB will be referred to as ‘Endogenous Control’, the gene of interest at treatment 0 mg Cu/L

(when referencing cell culture samples) or liver tissue is the 'Control Gene'. The gene of interest at all other Cu treatments is referred to as 'Treated Gene'.

The delta C_T values were calculated as

$$\Delta C_T = C_{T\ \text{Gene of Interest}} - C_{T\ \text{Endogenous Control}}$$

The delta C_T values were then calculated as

$$\Delta\Delta C_T = \Delta C_{T\ \text{Treated Gene}} - \Delta C_{T\ \text{Control Gene}}$$

The $2^{-\Delta\Delta C_T}$ method was used to calculate the relative quantification as

$$RQ = 2^{-(\Delta\Delta C_T)}$$

The relative quantification values were then log transformed as

$$\log(RQ,2)$$

STATISTICAL ANALYSIS

R software was used for the analysis of all data. Data were analyzed as a randomized, complete block design for both experiments. Treatment was included in the model as a fixed classification variable. Data was analyzed using the Type III ANOVA procedure in the 'car' package (Fox and Weisberg, 2011), a pairwise comparison was analyzed using gene by treatment using the emmeans procedure in the 'emmeans' package (Lenth et al., 2019), of R© (R Core Team, 2018). The effect of treatment was determined significant at $P \leq 0.05$.

RESULTS

Experiment 3: Table 3.4 shows the ΔC_T expression of Cu homeostatic genes in vitro relative to their abundance in an in vivo liver sample. The smaller the number, the less time it took for the C_T to be met. The relative abundance of mRNA associated with APOA1, ATOX1, ATP7A, ATP7B, BHMT, BLVRB, CA2, COX17, CTR1, GLUD1, and GSS related to hepatic Cu homeostasis were greater ($P < 0.001$) in liver tissue than in cell cultured hepatocytes. This table is supported by Figure 3.1: This figure shows a bar graph of the log transformed relative

abundance of the genes represented in Table 3.4. The log transformation indicates if the genes were up or down regulated when compared to a standardized sample. In Figure 3.1, all genes assessed, were down regulated relative whole liver.

Experiment 4: Table 3.5 shows the impact of Cu dose on the relative abundance of selected genes. All fourteen Cu homeostatic genes, ALDH2, ATOX1, ATP7A, ATP7B, BHMT, BLVRB, CA2, CCS, COX17, CTR1, GLUD1, GSS, PDIA3, and SOD1 showed at least one significant ($P < 0.05$) interaction across treatment. This table can be supported by Figure 3.2: This figure shows a bar graph of the log transformed relative abundance of the genes represented in Table 3.5. Some data points on the bar graph are missing due to insufficient data.

Table 3.1 shows the genes of interest for both experiments. Genes are grouped by their respective protein function when comparing results of qRT-PCR analysis. There were three general groups for selected genes: Cu requiring enzymes, intracellular transporters, and transmembrane transporters. Each gene within one of those three groups has a specific function which is outlined in the right column in Table 3.1. Table 3.2 shows cycle threshold (C_T) mean, standard deviation (SD) standard error of the mean (SEM) across eight commonly used endogenous controls. Endogenous controls were compared across both whole liver tissue and cell cultured hepatocytes and β-Actin (ACTB) was selected as the endogenous control for both experiments due to its small SD and SEM compared to the other endogenous controls. Table 3.3 shows forward and reverse primer sequences used in both experiments.

DISCUSSION

Using the methodology derived from Spotorno et al. (2006) with modifications, we achieved successful culture, dosing, and measurement of Cu homeostatic gene expression of hepatocytes derived from small samples of liver. Cell samples yielded lesser RNA than whole

liver tissue. All genes measured in this experiment were down regulated in cell culture samples when compared to liver tissue samples. This indicates the difference in relative abundance of Cu homeostatic mRNA from whole liver vs cultured hepatocytes. This should be considered when making comparisons reported in the literature. Cell cultured bovine hepatocytes were only at a cell live percentage of 70%. This initial cell death in extracted hepatocytes prior to addition of treatments could be an additional reason for down regulation of the relative abundance of mRNA from genes associated with Cu homeostasis. Hepatocytes dosed with 0, 0.1, 1.0, and 10.0 mg Cu/L yielded an average of 12 ng/µL of RNA. Relative abundance of mRNA associated with ALDH2 was similar at 0, 0.1, 1.0, but different at 10.0 and 100.0. Relative abundance of mRNA associated with ATOX1 was similar at 0, 0.1, 1.0, 10.0, but different at 100.0. Relative abundance of mRNA associated with ATP7A was similar at 0, 0.1, 1.0, but different at 10.0 and 100.0. Relative abundance of mRNA associated with ATP7B was similar at 0, 1.0, 100.0 but different at 0.1 and 10.0. Relative abundance of mRNA associated with BHMT was similar at 0, 0.1, 1.0, but different at 10.0 and 100.0. Relative abundance of mRNA associated with BLVRB was similar at 0, 0.1, 1.0, 100.0 but different at 10.0. Relative abundance of mRNA associated with CA2 was similar at 0, 0.1, 1.0, 100 but different at 10.0. Relative abundance of mRNA associated with CCS was similar at 0, 0.1, 1.0, but different at 10.0 and 100.0. Relative abundance of mRNA associated with COX17 was similar at 0, 0.1, 1.0, 10.0 but different at 100.0. Relative abundance of mRNA associated with CTR1 was similar at 0, 0.1, 1.0, 100.0 but different at 10.0. Relative abundance of mRNA associated with GLUD1 was similar at 0, 0.1, 1.0, 10.0 but different at 100.0. Relative abundance of mRNA associated with GSS was similar at 0, 0.1, 1.0, 10.0 but different at 100.0. Relative abundance of mRNA associated with PDIA3 was similar at 0, 0.1, 1.0, 10.0 but different at 100.0. Relative abundance of mRNA associated

with SOD1 was similar at 0, 0.1, 1.0, 10.0 but different at 100.0. For specific positive or negative trends in relative abundance compared to dose 0, at a treatment of 10 mg Cu/L, 6 Cu requiring enzymes were upregulated, 4 of them follow a similar trend in up/down expression. All but one gene was down regulated at treatment 100mg/L; this could be due to cell death at a toxic level of Cu. The two transmembrane transporters identified in these experiments follow a similar trend in expression. ATPase copper transporting alpha, as previously described is an intestinal transmembrane transporter and literature suggests the gene is present in heart, brain, lung, muscle, kidney, and pancreas tissues of human patients but is not present in liver tissue (Vulpe et al., 1993). However, ATP7A gene was identified in both liver tissue and cultured hepatocytes in these experiments. Copper chaperone for superoxide dismutase is down regulated at two low doses and upregulated at two high doses. Due to CCS's involvement in shuttling excess Cu to produce the antioxidant, this is possible reasoning for upregulation in expression at higher doses.

CONCLUSION

These data suggest that Cu homeostatic gene expression is altered by the process of culturing hepatocytes. The abundance of Cu homeostatic gene transcripts are altered in response to different Cu concentrations in culture. β-Actin was the most appropriate endogenous control for use in this experiment. The impact of Cu on the relative abundance of mRNA associated with Cu homeostatic genes investigated in these experiments was minimal in low dose of 0.10mg/L on cultured hepatocytes. However, higher doses showed a greater impact on relative abundance in both an up and down regulation. These changes are likely due to an antioxidant stress response to elevated concentrations of Cu. Further investigation is needed to understand the changes happening at lower concentrations of Cu.

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Table 3.1. Copper responsive genes of interest grouped by translated protein function

Genes of Interest	Acronym	Function
Aldehyde dehydrogenase	ALDH2	Cu requiring enzyme that binds to retinal proteins that may minimize Cu induced stress (Simpson et al., 2004)
Betaine homocysteine methyltransferase	BHMT	Cu requiring enzyme that synthesizes methionine (Simpson et al., 2004)
Flavin reductase	BLVRB	Cu requiring enzyme that protects cells from oxidative damage (Simpson et al., 2004)
Carbonic anhydrase II	CA2	Cu requiring enzyme known in hepatocytes to be glutathionylated during oxidative stress (Simpson et al., 2004)
Elastin	ELN	Cu requiring enzyme associated with oxidative stress (Han et al., 2012)
Glutamate dehydrogenase	GLUD1	Cu requiring enzyme involved in production of NADPH (Simpson et al., 2004)
Glutathione synthetase	GSS	Cu requiring enzyme that can lead to an inability to resist oxidative stress in deficient state (Simpson et al., 2004)
Lysyl oxidase	LOXL1	Cu requiring enzyme involved in ELN formation (Han et al., 2012)
Protein disulphide isomerase A3	PDIA3	Cu requiring enzyme that plays a role in protein folding (Simpson et al., 2004)
Superoxide dismutase [Cu-Zn]	SOD1	Cu requiring enzyme found to decrease in mRNA expression in Cu deficient liver (Suazo et al., 2008)
Extracellular superoxide dismutase	SOD3	Cu requiring enzyme with a dependent relationship with its Cu content (Jeney et al., 2005)
Apolipoprotein A-1	APOA1	Intracellular transporter involved in lipid metabolism and found to be down regulated in Cu challenged liver (Simpson et al., 2004)
Antioxidant 1	ATOX1	Intracellular transporter required for full activity of SOD enzymes (Jeney et al., 2005)
ATPase copper transporting beta	ATP7B	Intracellular transporter is involved in metabolizing Cu and incorporating Cu into CP (Suttle, 2012)
Copper chaperone for superoxide dismutase	CCS	Intracellular transporter important for shuttling Cu to SOD1 in the cytosol (Spears et al., 2011)
Cytochrome c oxidase copper chaperone	COX17	Intracellular transporter important for delivery of Cu to the mitochondria (Prohaska, 2008)
ATPase copper transporting alpha	ATP7A	Transmembrane transporter protein that transfers Cu from the intestine to the liver (Suttle, 2012)
Copper transporter 1	CTR1	Transmembrane transporter responsible for importing dietary Cu into the liver (Prohaska, 2008)

Table 3.2. Comparison of endogenous controls using bovine liver and primary cell cultured bovine hepatocytes treated with various doses of copper.

Endogenous Control	n	C_T Mean	SD	SEM
18S	21	15.09	2.08	0.45
ACTB	20	20.84	0.83	0.19
B2M	21	20.59	1.76	0.38
GAPDH	21	23.98	2.03	0.44
HMBS	20	29.76	1.94	0.43
HPRT1	21	26.48	1.99	0.43
RPLP0	19	21.09	1.07	0.25
RPS9	20	23.15	1.60	0.36

Table 3.3. Primer sequences for real time PCR

Primer	Forward	Reverse
ACTB	AGAGCTACGAGCTTCCTGAC	GCGCGATGATCTTGATCTTCATT
ALDH2	TCGGGAGAGCCAACAATTCC	TGACGGTCTTCACTTCGGTG
APOA1	GAGACTGCTGGCCATTGAGGT	TTGGGCCACATAGTCTCTGC
ATOX1	ACCTGCCCAACAAAAAGGTCT	CGCAGGAACTACCATTGCTGA
ATP7A	GTTGCTGGACCGAATTGTT	CAACGCACTGTCGTCATCTT
ATP7B	GTGGGCAATGATACGACCTT	CCATACCACCAACGTCACAG
BHMT	GGGGCAAAAATGTCAAGAAGGG	CCCTGTTCTCCAGCTTGTCTT
BLVRB	CCCAAGACTGCAGGATGTGA	TGTGCCCGTCGTACTTATCG
CA2	CTCGAAAGCGTGACCTGGAT	GCTAAGCTAAAGGGAGGCCC
CCS	TGTGGGGACCACTTTAACCC	CACACCTTTGAGGGGAGACA
COX17	TTTGGGGCACGAAAAATGCC	ACCATGCTCACCATTTCATATCTT
CTR1	GGGTACCTCTGCATTGCTGT	ATGGCAATGCTCTGTGATGT
ELN	AGCCAAGTCTGCTGCTAAGG	GTCCGAACTTGGCTGCTTTA
GLUD1	TGCCAAAGCTGGTGTGAAGAT	ACCCTGACTGATGGGCTTAC
GSS	CTGGACGACGGAAGAGTTGGG	TTGGGACCGGTGAAGGGAAG
PDIA3	CACTGCAAAAAGCTTGCCCC	TCAGGTGGCTGACAATTCCA
SOD1	AAGAGAGGCATGTTGGAGACC	CAGCGTTGCCAGTCTTTGTA
SOD3	CCGTGAAACCGTTTGAAGCA	CTGCATCATCTCCTGCCAGA
LOXL1	GTCCAGAGAGCCCACCTGTA	CATGCTGTGGTAGTGCTGGT

Table 3.4. The change (Δ) in cycle threshold (C_T) between bovine liver and hepatocytes cultured from bovine liver.

Item	Cell ΔC_T	Liver ΔC_T	SEM	P<
APOA1	7.83	0.28	0.203	0.0007
ATOX1	6.35	-0.26	0.417	0.0004
ATP7A	7.93	4.54	0.379	0.002
ATP7B	9.46	3.51	0.396	0.0005
BHMT	10.80	-0.89	0.089	0.00000001
BLVRB	3.85	-1.19	0.215	0.000008
CA2	8.64	6.25	0.124	0.00001
COX17	5.55	1.05	0.124	0.0008
CTR1	7.78	0.74	0.310	0.0001
GLUD1	6.22	-0.59	0.183	0.000001
GSS	9.69	5.88	0.528	0.0009

Table 3.5. The effects of copper (Cu) treatment on cultured bovine hepatocytes measuring change (Δ) in threshold (C_T)

Item	Treatment (ppm Cu)					SEM	P<
	0	0.1	1.0	10.0	100.0		
ALDH2	4.06[a]	3.57 [a,b,c]	3.76 [a,b]	3.15 [c]	3.54 [b,c]	0.107	0.0019
ATOX1	6.36 [a]	6.54 [a,b]	6.58 [a,b]	6.72 [a,b]	7.46 [b]	0.196	0.041
ATP7A	7.93 [a]	7.82 [a]	8.54 [a,b]	6.90 [c]	8.90 [b]	0.174	0.0001
ATP7B	9.46 [a]	8.20 [b]	10.06 [a]	7.34 [b]	10.50 [a]	0.224	0.00004
BHMT	10.12 [a,b]	10.69 [b]	10.51 [b]	8.87 [a]	11.26 [b]	0.291	0.002
BLVRB	3.85 [a]	3.78 [a]	4.13 [a]	3.25 [b]	4.18 [a]	0.107	0.0008
CA2	8.64 [a]	8.55 [a]	8.71 [a]	7.76 [b]	8.79 [a]	0.107	0.0003
CCS	10.9 [d,e]	11.9 [e]	11.9 [e]	10.4 [d]	10.3 [d]	0.293	0.03
COX17	5.55 [a]	5.78 [a]	5.48 [a]	5.36 [a]	6.36 [b]	0.117	0.0015
CTR1	7.79 [a]	7.66 [a]	8.31 [a]	6.49 [b]	8.27 [a]	0.161	0.0007
GLUD1	6.22 [a,b]	5.71 [a]	6.12 [a,b]	6.44 [b,c]	6.78 [c]	0.119	0.001
GSS	9.69 [a,b]	9.15 [a]	9.89 [a,b]	9.41 [a]	10.89 [b]	0.292	0.015
PDIA3	3.44 [a,b]	3.14 [a]	3.45 [a,b]	3.26 [a,b]	3.66 [b]	0.080	0.020
SOD1	4.90 [a]	4.96 [a]	5.07 [a,b]	4.91 [a]	5.46 [b]	0.097	0.012

[a,b,c]. Means within a row lacking a common superscript differ *P <0.05*.

[d,e.] Means within a row lacking a common superscript tend to differ *P <0.10*.

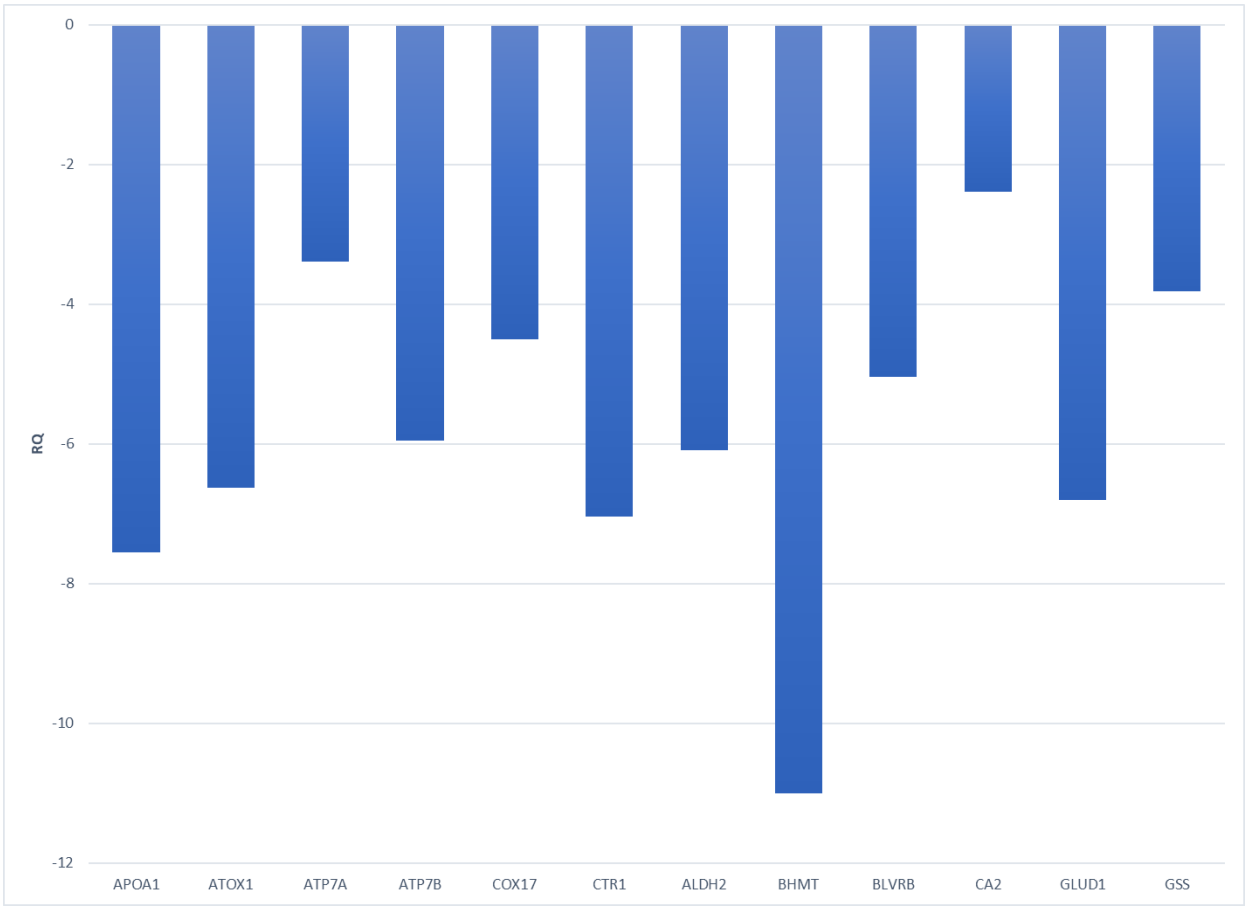

Figure 3.1: Log transformed relative abundance of cell cultured bovine hepatocytes compared to the liver they derived from adjusted to 1

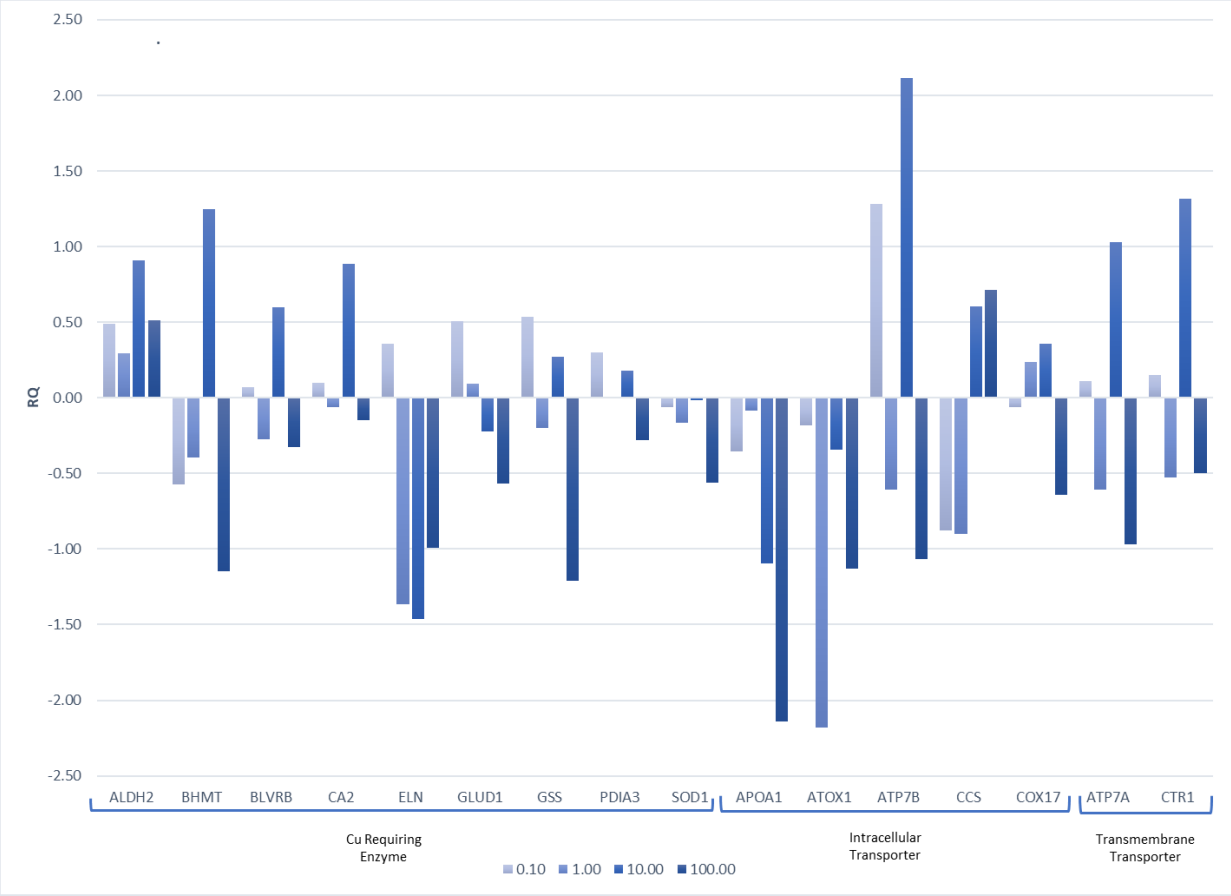

Figure 2.2: Influence of copper dose on log transformed relative abundance of copper homeostatic genes in cultured bovine hepatocytes

CHAPTER 4−INVESTIGATING THE INFLUENCE OF COPPER FED TO MULTIPAROUS BEEF COWS ON COPPER HOMEOSTATIC GENES IDENTIFIED IN LIVER SAMPLES

SUMMARY

Experiment 5: the objective of the final experiment was to compare gene expression of Cu homeostatic genes from a subset of bovine livers in a coexisting study investigating molybdenum (Mo) supplied in drinking water to primiparous cows. Liver samples were obtained from cows on two treatments within the experiment. Treatments consisted of: 1) no supplemental Cu (-Cu; total diet contained 7.0 mg Cu/kg DM) and 2) 3 mg Cu/kg DM (+Cu; total diet contained 10.0 mg Cu/kg DM). Livers were harvested immediately upon slaughter, rinsed with a phosphate buffered saline solution, diced into small pieces, and placed into RNA later solution. RNA was extracted from all samples in TRIzol™ and total RNA isolated. Quantitative RT-PCR was used to determine the abundance of transcripts for proteins involved in Cu homeostasis in liver tissue. The identified targets were: ALDH2, APOA1, ATOX1, ATP7A, ATP7B, BHMT, BLVRB, CA2, CCS, COX17, CTR1, ELN, GAPDH, GLUD1, GSS, LOXL1, PDIA3, SOD1, SOD3. Relative abundance of APOA1, ATOX1, ATP7B, BLVRB, CTR1, GLUD1, LOXL1, and SOD3 were greater ($P < 0.05$) in cows receiving supplemental Cu when compared to cattle not receiving supplemental Cu. Although protein expression from experiment 1 were not different in cattle receiving different Cu treatments, gene expression is different for certain Cu homeostatic genes. These results indicate a possible homeostatic difference between cattle supplemented Cu. Further investigation is warranted to determine if a difference in relative gene expression is associated with a difference in protein function.

INTRODUCTION

In previous experiments, we have reported differences in gene expression within whole liver and cell cultured hepatocytes. The main difference being that all copper (Cu) homeostatic

genes investigated in the experiment had lower abundance of mRNA present in cell culture indicating a difference between *in vitro* and *in vivo* methods. We have also reported differences in cultured liver cells when dosed with different concentrations of Cu. When does of Cu ranging from 0.10-100.0mg/L were added to cell cultured bovine hepatocytes for 1hr in culture, all Cu homeostatic genes of interest had at least one statistically significant difference in relative gene expression. Comparing all doses to the control (dose 0), dose 0.1 was only different for ATP7B. Across all genes measured, dose 1.0 was similar to 0. Dose 10.0 was different for ALDH2, ATP7A, ATP7B, BLVRB, CA2, CTR1. Dose 100.0 was different than 0 for genes ALDH2, ATOX1, ATP7A, BHMT, COX17, GLUD1, SOD1. Dose 100.0 had the most influence on Cu homeostatic genes. Therefore, the objective of this experiment was to investigate the impact of Cu supplementation to cows on the relative abundance of mRNA associated with genes involved in liver Cu homeostasis. The hypothesis of this experiment is that supplemental Cu in live cattle will influence Cu homeostatic genes in liver samples.

MATERIALS AND METHODS

A portion of the caudate lobe of liver from healthy multiparous cows was collected from at slaughter. Following collection, samples were rinsed using a phosphate buffered saline (PBS) solution. Samples were then cubed into small ~1.5cm pieces and placed into a 50mL falcon tube containing RNA*later*™ by Fisher Scientific LLC. (4500 Turnberry Drive Hanover Park, IL 60133) (catalog# AM7021). Samples were placed on ice and stored at 4°C for 3 days. Samples were then prepped for freezing and small pieces were aliquoted in 1.5mL cryovial and stored at -80°C. Total RNA was isolated from bovine liver tissue following a TRIzol™ reagent protocol. In brief, the steps of the protocol included: homogenization, phase separation, RNA precipitation, wash, and solubilization (Chomczynski, 1993). For tissue samples, on average 60 mg was added to a 2.0 mL 0.8mm garnet bead tube with 1 mL TRIzol™ reagent from Fisher

Scientific LLC. (Catalog #15596018). Samples were homogenized with a Bead Bug Microtube Homogenizer from Benchmark Scientific (2600 Main Street Extension, Sayreville, NJ 08872). The DNAse treatment was conducted with a TURBO DNA-free Kit. Concentrations and purity were estimated with a NanoDrop™ One Microvolume UV-Vis Spectrophotometer from Fisher Scientific LLC. (4500 Turnberry Drive Hanover Park, IL 60133). Post DNA-se treatment, liver samples averaged 4400 ng/µL. All liver samples were normalized to 500 ng/µL. Samples were then converted to cDNA using a standard protocol and combination of random hexamer and oligo-dT primers. Post cDNA conversion, liver samples were diluted using equal parts water to cDNA volume and like samples were pooled and aliquoted to minimize freeze thaw cycles. Endogenous control ACTB was selected from a previous experiment and used as the best fit endogenous control for liver samples.

STATISTICAL ANALYSIS

R software was used for the analysis of all data. The experimental design was a randomized, complete block design with 2 treatments. The relative abundance of mRNA associated with genes involved in liver Cu homeostasis and ΔC_T values were analyzed consider response variables and treatment was included in the model as a fixed classification variable. Data was analyzed using the Type III ANOVA procedure in the 'car' package (Fox and Weisberg, 2011), a pairwise comparison was analyzed using gene by treatment using the emmeans procedure in the 'emmeans' package (Lenth et al., 2019), of R© (R Core Team, 2018). Significance was determined when $P \leq 0.05$.

RESULTS

Table 4.1 shows the change (Δ) in cycle threshold (C_T) calculated by subtracting the C_T of interest from the C_T of the endogenous control ACTB. Values expressed were not log transformed, so the smaller the value shown in the table, the sooner it reached threshold,

therefore the smaller the value the higher relative abundance of mRNA. These results confirm the values represented in Figure 4.1. Figure 4.1 shows the log transformed relative quantification of Cu homeostatic genes for Treatment 2 (+Cu) when normalized against Treatment 1(-Cu). The results indicate that APOA1, ATOX1, ATP7B, BLVRB, CTR1, GLUD1, LOXL1 are in greater abundance in +Cu treatment when compared to -Cu treatment. Furthermore, SOD3 has a lesser abundance in +Cu treatment when compared to -Cu treatment. Although COX17 and ELN appear to be significantly different, there were values omitted from both due to insufficient PCR readings.

DISCUSSION

Although the liver Cu concentrations were not significantly different, the cattle were on feed for 419 days and samples were collected after they had been off the treatments for 121 days. This explains why the livers had normalized to a common Cu concentration. Since genes involved in Cu homeostasis were still different after 121 days, these data indicate long tern impacts of Cu supplementation Cu homeostatic genes. Flavin Reductase (BLVRB), GLUD1, and SOD1 were categorized as Cu requiring enzymes and are possibly upregulated in this experiment to produce more antioxidants to counter the influx of Cu in a reactive state. The relative abundance of mRNA for ATOX1was upregulated, ATOX1 function is to shuttle Cu to ATP7B. This is possibly upregulated because that pathway is responsible for Cu excretion and treatment 2 received a greater amount of dietary Cu. The function of CTR1 is to bring Cu into the liver cell. The relative abundance of mRNA of CTR1 is possibly upregulated to allow more Cu from the diet in to metabolize and excreted quicker. The gene LOXL1 is involved in ELN production. Both LOXL1 and ELN were down regulated so it is possible that production of ELN was reduced in order to handle the antioxidant stress from increase Cu, resulting in both genes having

lower expression for the animals fed Cu. The experiment by Simpson et al., (2004) found that the protein expression of SOD3 had a dependent relationship with Cu. In their experiment, as Cu content increased, so did the expression of SOD3 (Simpson et al., 2004). Our results indicate that relative abundance of mRNA for SOD3 is down regulated in the Cu treated liver samples. This difference may indicate that up or down regulated relative abundance of mRNA is not indicative of the protein expression that the gene codes for.

CONCLUSION

Relative expression of mRNA in BLVRB, GLUD1, LOXL1, SOD1, SOD3, APOA1, ATOX1, ATP7B, CTR1 Cu homeostatic genes differed in +Cu treatment compared to -Cu treatment. We failed to reject our hypothesis that Cu homeostatic genes differ in animals supplemented Cu in their diet. Further investigation is needed to determine if an up or down regulation of Cu homeostatic genes in liver tissue dictates liver protein function.

LITERATURE CITED

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Table 4.1. The effects of copper (Cu) supplemented liver copper concentration on change (Δ) in threshold (C_T)

Item	Treatment 1 No supp. Cu ΔC_T Mean	Treatment 2 3mg Cu/kg DM ΔC_T Mean	SEM	P<
Liver Cu Concentrations, mg/kg DM	7.63	7.30	1.86	0.862
Aldehyde dehydrogenase (ALDH2)	-0.74	-1.06	0.216	0.16
Apolipoprotein A-1 (APOA1)	1.57	1.15	0.144	0.01
Antioxidant 1 copper chaperone (ATOX1)	1.13	0.52	0.252	0.03
ATPase copper transporting alpha (ATP7A)	7.22	7.23	0.096	0.92
ATPase copper transporting beta (ATP7B)	4.17	3.5	0.198	0.005
Betaine homocysteine methyltransferase (BHMT)	0.62	0.34	0.241	0.26
Flavin reductase (BLVRB)	1.44	0.84	0.224	0.02
Carbonic anhydrase II (CA2)	8.10	7.94	0.250	0.51
Copper chaperone for superoxide dismutase (CCS)	9.15	9.63	0.416	0.26
Cytochrome c oxidase copper chaperone (COX17)	2.28	3.93	1.012	0.12
Copper transporter I (CTR1)	2.34	1.92	0.197	0.03
Elastin (ELN)	10.23	10.90	0.298	0.05
Glutamate dehydrogenase (GLUD1)	1.76	0.83	0.273	0.003
Glutathione synthetase (GSS)	5.53	5.40	0.185	0.49
Lysyl oxidase (LOXL1)	5.06	5.50	0.163	0.02
Protein disulphide isomerase A3 (PDIA3)	1.05	1.26	0.295	0.49
Superoxide dismutase [Cu-Zn] (SOD1)	0.79	0.17	0.205	0.008
Extracellular superoxide dismutase (SOD3)	5.81	6.63	0.338	0.02

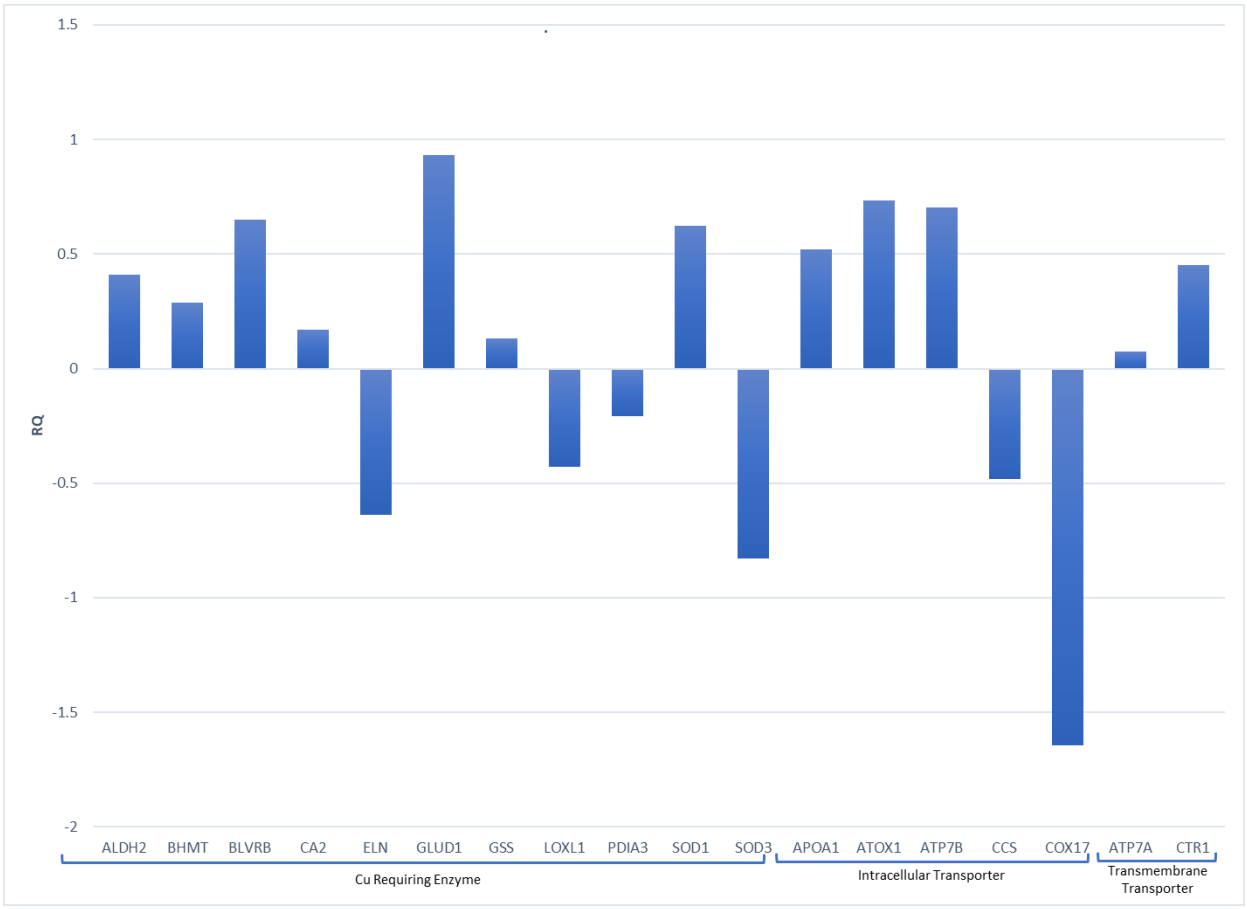

Figure 3.1: Log transformed relative gene expression between bovine liver fed Cu compared to a negative control