EFFECT OF ALCOHOL INGESTION ON PROTEINS INVOLVED IN IRON TRANSPORT IN MOUSE DUODENUM

DISSERTATION

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Department of Biochemistry, 29th September, 2010

Christian Medical College,

Vellore-632002

This is to certify that this study entitled “Effect of alcohol ingestion on proteins involved in iron transport in mouse duodenum” is the bonafide work of Dr. Subhosmito Chakraborty, who conducted it under my guidance and supervision. The work in this dissertation has not been submitted to any other university for the award of a degree.

Dr. Molly Jacob, M.D., Ph.D.

Professor and Head,

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Christian Medical College,

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DECLARATION

I hereby declare that the investigations which form the subject matter of this study were conducted by me under the supervision of Dr Molly Jacob, Professor of Biochemistry, Christian Medical College, Vellore.

Dr Subhosmito Chakraborty

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28.09.2010

To

The Controller of Examinations,
The Tamil Nadu Dr. M.G.R. Medical University,
40, Anna Salai, Guindy,
Chennai 600 032.

Dear Sir,

This is to certify that the study "Effect of alcohol ingestion on proteins involved in iron transport in mouse duodenum" by Dr Subhosmito Chakraborty was approved by the Institutional Animal Ethics in consultation with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) (IAEC No 7/2009 dated 27th May, 2009).

Thanking you,

Yours sincerely,

Dr. Solomon Sathiskumar
Secretary - Institutional Animal Ethics Committee

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INTRODUCTION:

Iron is an essential micronutrient in the human body. It serves several important functions. It is a part several proteins, such as hemoglobin, myoglobin and cytochromes (Andrews and Schmidt, 2007). A 70-kg man has a body iron content of approximately 3.5g of iron. Of this, about 65% (2.3g) is found in hemoglobin in erythrocytes and 10% in myoglobin in muscles (350mg). The rest is found in the reticulo-endothelial system (500mg), liver (200mg) and bone marrow (150mg) (Munoz et al., 2009). Pre-menopausal women have lower stores of iron than men. The average diet provides about 15-20 mg of iron per day, but only 1-2 mg is absorbed. This restores equivalent amounts of iron lost from the body by sloughing of the intestinal mucosa and menstrual blood losses in women. The major demand for iron, chiefly by the erythrocytes, is met by internal recycling of iron (15-20 mg/day) by splenic macrophages (Andrews, 1999). There is no effective means of excretion of iron from the body. Hence, the regulation of dietary iron absorption in the duodenum plays a critical role in iron homeostasis (Siah et al., 2006)

ABSORPTION OF IRON FROM THE GUT:

Iron is available to animals, mainly from dietary sources, either in elemental (inorganic) form or as heme. Absorption of dietary inorganic iron takes place predominantly in the duodenum. The absorptive cells, the enterocytes, display polarity, as their apical microvilli brush border surface is separated from their basolateral surfaces by tight junctions. There is little evidence of paracellular iron transport, so each step of iron transfer from the gut to the circulation requires dedicated proteins, aided by several enzymes that alter the oxidation states of iron as required by the various proteins involved (Andrews and Schmidt, 2007) (Figure1).

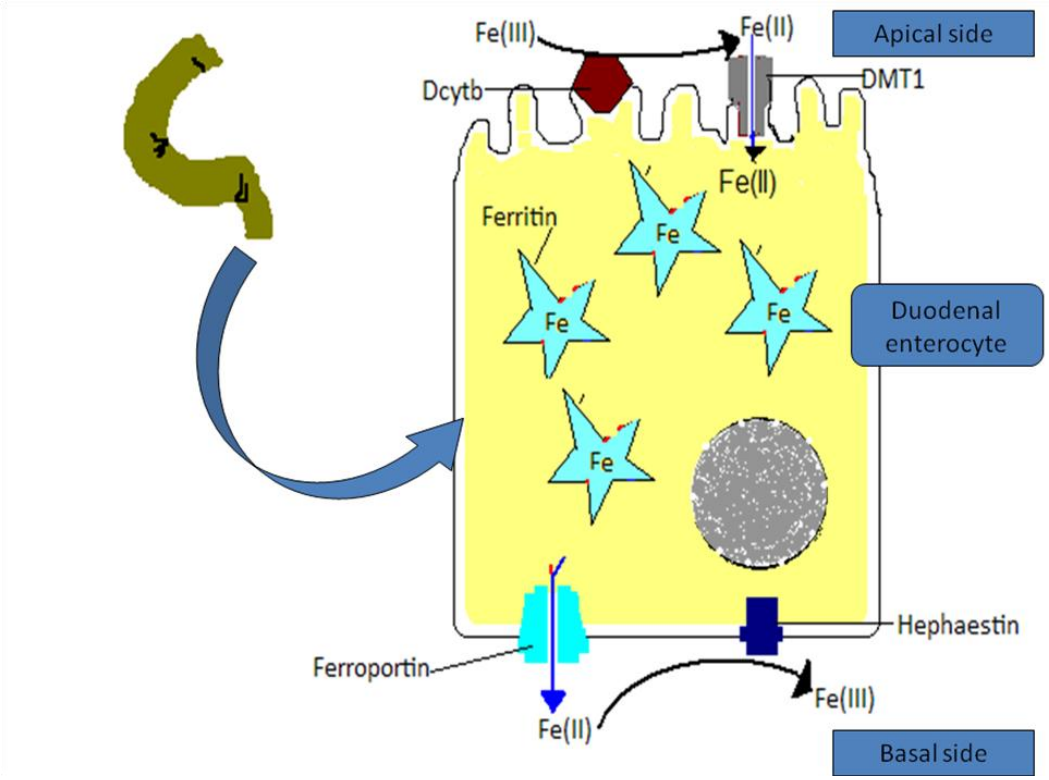

Figure1: The various proteins involved in iron absorption in duodenal enterocytes. The function of each protein and its location are detailed in the text below.

Absorption of dietary inorganic iron:

Duodenal enterocytes have a specialized transport protein called divalent metal transporter 1 (DMT-1) on their apical surface. This protein, also called SLC11A2, NRAMP2 or DCT 1, transports ferrous iron from the intestinal lumen into enterocytes (Gunshin et al., 1997). It is a 12-transmembrane segment protein and a proton symporter (Gunshin et al., 1997). It requires a low pH for efficient iron transport. A spontaneous mutation in DMT1 (G185R), identified in Belgrade b rats with microcytic anemia phenotype, has offered insights into the in-vivo functioning of this transporter (Fleming et al., 1997, Fleming et al., 1998). The G185R mutation converts the channel of this protein to an L-type calcium channel (Xu et al., 2004). Knock-out murine models (slc11a2-/- mice) have confirmed the role of this transporter in intestinal iron absorption and uptake by erythroid tissue (Gunshin et al., 2005). Human patients, with DMT1 mutations, have been reported to have congenital anemia (Beaumont et al., 2006, Iolascon et al., 2006, Mims et al., 2005).

In the diet, iron is largely found in its ferric state. It needs to be reduced to its ferrous form for absorption to occur. This has been shown to be carried out by a membrane-associated ferri-reductase, known as duodenal cytochrome b (dcytb/Cybrd), on the basis of its homology to a cytochrome family protein b561(Oakhill et al., 2008). It is thought to utilize vitamin C as a reductant. DcytB levels have been shown to be increased in iron deficiency anemia (McKie et al., 2001). However, dcytb knockout mice do not show any abnormalities of iron metabolism, indicating that dcytb is not indispensible for iron metabolism (McKie, 2008). No mutations in dcytB have been documented in humans. Other putative reductases are that have been identified comprise the STEAP family of proteins (Ohgami et al., 2006).

Absorption of heme iron:

Heme, as a dietary source of iron, is found in hemoglobin or myoglobin in meat in the diet. The exact mechanism involved in the absorption of heme in the gut is not clear. SLC46A1, also known as heme carrier protein1 (HCP1), which was initially proposed as a heme transporter (Shayeghi et al., 2005), has since been shown to carry mostly folate (Qiu et al., 2006). Once inside the enterocytes, iron present in heme is released by heme oxygenase 1 (HOX1) (Ferris et al., 1999).

Export of iron from the enterocytes:

Depending on the requirements of the body, iron absorbed from the diet is either exported out into the circulation or remains stored as ferritin in enterocytes. Sloughing of the intestinal mucosa, which occurs every 2-3 days, results in loss of iron stored in enterocytes. Iron is exported from enterocytes (and other cells) by ferroportin (FPN; also known as IREG1, SLC40A1, MTP1), found on the basolateral membranes of the cells. Studies on knockout mice (slc40a1-/-) have revealed some aspects of its functioning (Donovan et al., 2005). It has been proposed to have 10-12 transmembrane segments (McKie et al., 2000, Abboud and Haile, 2000, Donovan et al., 2000). It exports ferrous iron, which is subsequently oxidized to ferric iron, for transport by transferrin in blood. This oxidation is carried by ceruloplasmin in most cells and hephaestin in the duodenum (Hower et al., 2009). Hephaestin, an enzyme with ferroxidase activity that is analogous to

ceruloplasmin, oxidizes iron to the ferric form (Vulpe et al., 1999) Hephaestin-deficient mice have been shown to have iron deficiency anemia, with associated mucosal retention of iron (Andrews and Schmidt, 2007).

TRANSPORT OF IRON IN BLOOD:

Under physiological circumstances, most of the iron in circulation is carried by transferrin (TF). Only a negligible amount is bound to albumin. In humans, TF is usually 30% saturated with iron; in rodents, this figure goes up to 60-80%. A diurnal variation in TF saturation has been reported (Uchida et al., 1983). TF saturation has also been found to show regional differences; it is higher in periportal hepatocytes, where newly absorbed iron arrives first and it is lower in blood leaving erythroid tissue, which is the largest consumer of iron (Andrews and Schmidt, 2007). In humans, a saturation level of less than 16% indicates iron deficiency, while a saturation level of more than 45% indicates iron overload. When saturation levels exceed 60%, non-transferrin-bound iron (NTBI) begins to accumulate in liver and causes parenchymal damage(Hentze et al., 2010).

CELLULAR UPTAKE OF TRANSFERRIN-BOUND IRON

Cells in the body express transferrin receptors (TfR). They are responsible for uptake of iron by receptor- mediated endocytosis (Hentze et al., 2004). After endocytosis, the endosome formed is acidified to a pH of 5.5 through the action of a proton pump. TF and TfR undergo conformational changes, leading to release of iron. The ferric iron released from TF must be reduced to ferrous iron for transport by DMT1 out of the endocytic vesicle. Elegant genetic experiments suggest that STEAP3 serves this function (Ohgami et al., 2005) in all cells, except for enterocytes, where dcytb carries out this function. A mouse mutant lacking STEAP3 has been shown to develop iron-deficiency anemia, attributable to a defect in endosomal ferrireductase function (Ohgami et al., 2005).

Even though TfR is expressed widely in tissues, non-hematopoeitic tissues can use non-TfR-dependent mechanisms to assimilate iron (Syed et al., 2006). TfR knockout (TfR-/-) mice die in the

embryonic stage, with death occurring at 11.5 days on an average (Levy et al., 1999a). Death is attributable to severe anemia in the embryo. Non-hematopoietic tissues in these mice do not show damage, except in neuro-epithelial cells (which undergo apoptosis) and the neural tube (which may be kinked).

Human mutations in TfR have not been reported till date but antibodies against TfR are seen in severe cases of acquired anemia (Larrick and Hyman, 1984). Spontaneous mutations in transferrin have been documented in humans (Beutler et al., 2000, Asada-Senju et al., 2002) and animals (Bernstein, 1987, Trenor et al., 2000). Profound deficiency of transferrin results in severe anemia and central nervous system abnormalities (Dickinson and Connor, 1994). The erythroid system develops an iron deficiency whereas other tissues a massive iron overload (Craven et al., 1987). This underscores the importance of the transferrin -TfR endocytic cycle in the erythron, and the fact that non-hematopoietic tissues accumulate iron via non- transferrin-mediated mechanisms. In such cases with transferrin mutations, intestinal iron uptake is increased, probably to compensate for the deficiency of iron in the erythron. This also highlights a signalling mechanism between the erythroid precursors and the intestine that regulates iron absorption (Finch, 1994).

CELLULAR UTILIZATION OF IRON:

Erythroid bone marrow is the largest consumer of iron. About 66% of the body's total iron content is located in erythroid precursor cells and mature red blood cells. About a billion iron atoms are used each day to synthesize new red blood cells.

Once iron leaves the endosome, it moves to the mitochondria for incorporation into protoporphyrin IX by ferrochelatase to form heme. Heme biosynthesis begins and ends in the mitochondrion, but some intermediate steps occur in the cytoplasm (Ponka, 1997). Mitoferrin (SLC25A37 or MFRN) is a protein involved in mitochondrial iron import (Shaw et al., 2006). This process is facilitated by the ABCB10 (ATP-binding cassette, subfamily B, member 10) protein, which is thought to stabilize MFRN1 (Chen et al., 2009). Zebrafish and mice lacking MFRN1 fail to incorporate erythroid iron into

heme. Iron may also be directly transported from endosomes into mitochondria by a “kiss-and-run mechanism” through a direct contact between both organelles, effectively bypassing the cytosol (Sheftel et al., 2007). In addition, Quigley et al (2004) have reported that a protein capable of cellular heme export, FLVCR, plays a key role in erythropoiesis The role of FLVCR in other tissues is, however, yet to be fully defined.

RECYCLING OF IRON:

Only a fraction of the iron bound to transferrin is from dietary iron. Most of it is derived from recycling of iron within the body. Old and damaged erythrocytes are phagocytosed by tissue macrophages, particularly in the spleen. The cells are lysed and hemoglobin is catabolized to liberate iron. Some of the iron remains in storage in macrophages and the rest is exported by ferroportin to bind to plasma transferrin. Ferroportin has been shown to be critical for macrophage iron export (Donovan et al., 2005) and can be regulated to change the ratio between stored and released iron.

INTRA-CELLULAR IRON BALANCE:

Cellular iron homeostasis is tightly regulated, as an adequate amount is needed for cellular functioning. Cells accomplish this by at least two mechanisms. First, all mammalian cells produce ferritin, an iron- storage protein. Ferritin is a 24-subunit polymer composed of varying ratios of two similar polypeptide chains, L-ferritin (light ferritin) and H-ferritin (heavy ferritin) (Theil, 2003). H-ferritin has oxidase activity. Ferritin polymers are cage-like structures, with a central cavity to store hydrated iron oxides. Each ferritin polymer can accommodate up to 4500 iron atoms. Ferritin acts as a depot, accepting excess iron and allowing iron mobilization when needed.

The second protective mechanism involves iron regulatory proteins (IRPs). These can bind to iron regulatory elements (IREs) found in the un-translated regions (UTR) of mRNAs that code for proteins involved in iron transport and storage. This occurs when iron levels are low. IRPs exist in 2 forms - IRP1 and IRP2. IRP1 can incorporate an iron-sulfur cluster (Fe-S), which acts as an iron

sensor (Rouault et al., 1991). In the presence of high levels of iron, the 4Fe-4S cluster is complete. Under these circumstances, IRP1 cannot bind RNA and assumes the function of cytoplasmic aconitase (Kaptain et al., 1991). IRP2 does not contain an iron-sulfur cluster, but it is rapidly ubiquinated and degraded in the presence of excess iron (Iwai et al., 1998). In the presence of low levels of iron, IRPs bind to IREs of mRNA for proteins involved in iron metabolism. This binding serves to either decrease or increase translation of these proteins. The effect produced depends upon the location of the IREs in the respective mRNAs (Pantopoulos, 2004). IRP binding to IREs found in the 5' untranslated regions of mRNAs encoding ferritin, ferroportin, and the heme biosynthetic enzyme aminolevulinate synthase sterically blocks the initiation of translation by interfering with ribosome assembly at the start codon. In the case of ferritin, this interrupts the production of both ferritin subunits. . IREs found in the 3' untranslated region of TfR1 mRNA serve a very different function. There, 5 tandem IREs bind IRP to stabilize the molecule by inhibiting nuclease digestion. This allows more TfR1 to be produced when iron is limiting. A single 3' UTR, presumably serving a similar function, is found in DMT1 mRNA (Andrews and Schmidt, 2007).

Mice deficient in IRP1 (Ireb -/-) do not show any phenotypic alterations (Meyron-Holtz et al., 2004) as compared to mice deficient in IRP2 function (Ireb2 -/-), which display severe abnormalities of iron homeostasis and neurodevelopment (LaVaute et al., 2001). No human mutations have been identified in these two IRPs till date.

REGULATION OF IRON ABSORPTION FROM ENTEROCYTES:

Because iron cannot be excreted from the organism in a regulated way, iron absorption has to be a critically controlled process. Normally, only 1–2 mg of iron per day are absorbed to compensate for iron losses through sloughing of intestinal epithelial cells, desquamation of skin and urinary cells, blood loss, sweat and menstrual losses in pre-menopausal women. Iron absorption can be enhanced when the needs are higher (for example, because of increased erythropoiesis or pregnancy) and suppressed in iron overload. The lack of an active mechanism for iron excretion

accounts for the development of iron overload when the regulation of iron absorption is defective or bypassed (as occurs in blood transfusions).

REGULATION OF SYSTEMIC IRON BALANCE:

Iron balance must be strictly regulated to provide iron as needed and to avoid the toxicity associated with iron excess. This starts at the level of intestinal absorption and also involves recycling of iron in macrophages and hepatocyte iron mobilization. Major stimuli known to modulate iron homeostatic mechanisms are erythroid iron needs, iron deficiency, hypoxia, iron overload and inflammation. Hepcidin, a circulating peptide hormone, is responsible for this regulation (Nicolas et al., 2001, Park et al., 2001, Pigeon et al., 2001).

Role of hepcidin in regulation of iron homeostasis:

Hepcidin is a 25-amino-acid protein that is primarily produced by hepatocytes and derived from an 84-amino-acid precursor. It has 8 cysteine residues, similar to anti-microbial defensin proteins, and has measurable anti-microbial activity. A hair-pin structure has been determined for hepcidin (Hunter et al., 2002). It has been reported that a copper atom may be associated with the cysteine residues (Melino et al., 2005).

Hepcidin is primarily produced by the liver but can also be produced by the heart, pancreas, and hematopoietic cells (Ilyin et al., 2003, Peyssonnaux et al., 2006). There are two hepcidin genes in mice, likely owing to a recent gene duplication event. Only mouse hepcidin 1 appears to have biological activity (Lou et al., 2004).

Several studies have documented the importance of hepcidin in iron metabolism. Hepcidin expression is increased in the liver of iron-overloaded animals (Pigeon et al., 2001). Nicolas et al. (Nicolas et al., 2001) showed that an incidental inactivation of the hepcidin gene led to an iron overload phenotype. Subsequent studies have reported that expression of a hepcidin transgene causes anemia by interrupting intestinal iron absorption and inducing the retention of iron within

recycling macrophages (Nicolas et al., 2001). Targeted disruption of the hepcidin gene itself causes severe iron overload in mice (Viatte et al., 2005). There are many stimuli which can alter hepcidin expression. It is known to be increased in response to increased serum iron, inflammation and diminished in response to increased erythroid drive, hypoxia, and iron deficiency (Nicolas et al., 2002b, Weinstein et al., 2002).

Studies in cultured cells have shown that hepcidin binds directly to ferroportin, causing its internalization and degradation within lysosomes (Nemeth et al., 2004b). Through this elegant mechanism, hepcidin directly regulates cellular iron release in enterocytes and macrophages. It is not clear if hepcidin regulates ferroportin activity in other cell types. In particular, no definitive role has been established for either ferroportin or hepcidin in iron mobilization from hepatocyte stores.

Factors that regulate hepcidin expression:

There are two types of TfRs expressed on plasma membranes. TfR1 is ubiquitously expressed , while TfR2 is expressed in only expressed on hepatocytes (Trinder and Baker, 2003). TfR1 is involved in iron uptake in hepatocytes from TF-Fe in circulation whereas TfR2 appears to act as an iron sensor (Robb and Wessling-Resnick, 2004, Johnson and Enns, 2004) rather than being involved in iron uptake. Its affinity for TF-Fe is lower than that of TFR1 (Hentze et al., 2010). Another protein called HFE (high iron) is necessary for functioning of TfR1 (Knutson, 2010). Transferrin-Fe and HFE both bind to TfR1 but their binding sites overlap resulting in a competition for binding. TfR2 on the other hand binds to transferrin-Fe at two distinct sites (Gao et al., 2009). When transferrin-bound iron increases in circulation, TfR1 becomes saturated causing HFE to be displaced from it and subsequently interact with TfR2 (Hentze et al., 2010). The HFE-TfR2 complex then activates hepcidin transcription, via ERK/MAPK and bone morphogenetic protein (BMP)/SMAD signaling (Hentze et al., 2010). The BMP co-receptor, hemojuvelin (HJV), interacts with type I and type II BMP receptors (BMPR) on the plasma membrane to induce phosphorylation of receptor-activated SMAD (R-SMAD) proteins, and subsequent formation of active transcriptional complexes involving the co-SMAD factor, SMAD4 (Hentze et al.). HJV is a homologue of repulsive guidance molecule proteins

important in neurodevelopment (Niederkofler et al., 2004). It is mutated in patients with severe, early-onset juvenile hemochromatosis. BMP-mediated signaling is inhibited by soluble HJV (sHJV). sHJV is probably cleaved from its membrane attachment by TMPRSS6, a serine protease, and released into the circulation. The exact location of this cleavage process is unknown. sHJV achieves inhibition of BMP-mediated signalling by acting as a decoy receptor for BMP6, preventing the interaction of BMP6 with its receptor (Knutson). SMAD7 interferes with SMAD4-controlled hepcidin activation (Arndt et al., 2010). The mechanism involved is not known.

It has been shown that liver-specific inactivation of the co-SMAD protein, SMAD4, causes a failure of hepcidin production and an iron overload phenotype similar to that observed in hepcidin knockout mice (Wang et al., 2005). On the other hand, hepcidin production is increased by treatment with BMPs (Wang et al., 2005, Babitt et al., 2006). There are several types of BMPs, but BMP6 plays the major signaling role in hepcidin expression (Arndt et al., 2010). The source of BMP6 in circulation has been shown to be in the small intestine in mice (Arndt et al.).

A second type of transcriptional regulation of hepcidin occurs in inflammatory conditions. Interleukin-6 (IL-6) and possibly other inflammatory cytokines induce transcription of the hepcidin gene in hepatocytes, both in vitro and in vivo (Nemeth et al., 2004a). This induction involves the activation of STAT3 (signal transducer and activator of transcription 3) and binding of STAT3 to a regulatory element in the hepcidin promoter (Wrighting and Andrews, 2006).

A third type of regulation occurs by erythropoietic factors. Erythropoiesis requires considerable quantities of iron, and the inhibition of hepcidin expression by erythropoietic signals is, thus, of great physiological importance. Nonetheless, the molecular mechanisms and factors responsible are still poorly understood. Hepcidin suppression in response to phlebotomy or hemolysis depends on intact erythropoietic activity in mouse models, with irradiation and cytotoxic inhibition of erythropoiesis preventing hepcidin suppression (Pak et al., 2006). Growth differentiation factor 15 (GDF15) and twisted gastrulation factor 1 (TWSG1) are both released by erythroid precursors. High doses of

GDF15 are detectable in the serum of patients with ineffective erythropoiesis, such as in β-thalassemia (Tanno et al., 2009). Such pathological concentrations of GDF15 can suppress hepcidin transcription in cell models, but the underlying molecular mechanism has not yet been characterized. By contrast, lower GDF15 concentrations fail to suppress hepcidin in cellular models and are apparently ineffective in patients with sickle cell anemia, myelodysplastic syndrome, and anaemia of chronic disease (ACD). TWSG1 expression is increased in thalassemic mice, where it is produced during early erythroblast maturation. In cellular models, TWSG1 inhibits BMP-dependent activation of SMAD-mediated signal transduction that leads to hepcidin activation (Tanno et al., 2009). Correlations between TWSG1 expression, serum iron parameters, and hepcidin levels have not yet been studied in human anemias.

Hypoxia also controls hepcidin expression. Liver-specific stabilization of the hypoxia-inducible factors 1 and 2 (HIF1 and HIF2) decreases hepcidin expression while chemical HIF stabilizers suppress hepcidin mRNA expression in hepatoma cells (Peyssonnaux et al., 2007). These findings have raised the possibility that iron-dependent prolyl hydroxylases involved in HIF degradation may act as hepatic iron sensors. It is not known whether HIF binds to the hepcidin promoter.

In vivo, hypoxia induces erythropoietin (EPO) synthesis, which in turn stimulates erythropoiesis. EPO injection into mice reduces hepcidin levels in a dose-dependent manner and can over-ride signals that activate hepcidin expression (Pinto et al., 2008). Even low dose EPO injections in human volunteers were found to decrease urinary excretion of hepcidin (Robach et al., 2009). Experimental blockade of erythropoietic activity was found to prevents the effect of EPO on hepcidin, suggesting that EPO probably suppresses hepcidin by stimulation of erythropoiesis (Pak et al., 2006).

DISORDERS ASSOCIATED WITH IRON OVERLOAD:

Hereditary iron overload disorders (hemochromatosis) have provided important insights into genes that regulate iron homeostasis. Similar clinical phenotypes of varying severity result from

homozygosity for mutations in HFE, transferrin receptor 2 (TfR2), Hjv, and hepcidin genes (Feder et al., 1996, Camaschella et al., 2000, Papanikolaou et al., 2004, Roetto et al., 2003). A related, autosomal dominant disorder is caused by mutations in ferroportin (Montosi et al., 2001, Njajou et al., 2001) that disrupt its regulation by hepcidin (De Domenico et al., 2006, Drakesmith et al., 2005, De Domenico et al., 2005). Affected patients with hereditary hemochromatosis (HH) have parenchymal iron deposition in the liver, heart, and endocrine tissues but a paucity of iron in intestinal epithelial cells and tissue macrophages (Fillet et al., 1989). In severe cases, tissue iron leads to cirrhosis, cardiomyopathy, diabetes and other endocrinopathies.

HH is caused due to mutations in the high iron gene (Hfe) (Feder et al., 1996, Feder et al., 1997). The HFE protein is a member of the major histocompatibility complex (MHC). Mutations in this gene disrupt its interaction with β2-microglobulin (Lebron et al., 1998). HH has a prevalence of 1 in 200-400 (Eijkelkamp et al., 2000). Depending on the population studied, a carrier rate of 1 in 7 to 1 in 10 has been reported (Eijkelkamp et al., 2000). The most common mutation seen is C282Y. C282Y disrupts HFE conformation but does not entirely prevent its function (Levy et al., 1999b). Another mutation that occurs is H63D (Waheed et al., 1997, Merryweather-Clarke et al., 1997). The discovery that HFE forms a protein-protein complex with TfR led to several hypotheses for its role in iron homeostasis (Roy and Enns, 2000). However, recent results suggest that the HFE/TfR complex itself does not have a direct iron-regulatory activity; rather, TfR appears to sequester HFE to prevent it from acting elsewhere (Andrews and Schmidt, 2007).

TfR2 encodes a homolog of TfR (Kawabata et al., 1999). It can bind and internalize Fe-Tf but probably does not serve a primary role in cellular iron uptake. Rather, it likely acts as an iron sensor; TfR2 protein levels increase in response to increased ambient Fe-TF (Robb and Wessling-Resnick, 2004, Johnson and Enns, 2004).

Investigations of the effect of alcohol on iron metabolism began in patients with genetic disorders of iron overload. . Consumption of alcohol by patients with HH was observed to exacerbate liver injury compared with those who did not consume excess alcohol, as shown by histopathological

examination of on the liver (Fletcher et al., 2002). HH livers showed extensive fibrosis (Fletcher et al., 2002).

Patients with HfE, TfR2, and ferroportin mutations typically present in mid-life. Most patients with HFE hemochromatosis never develop clinical disease, and it has a low clinical penetrance (Ajioka and Kushner, 2003, Waalen et al., 2002). In contrast, patients with Hjv and hepcidin mutations are severely affected early in life, typically dying of cardiomyopathy before the fourth decade, if not treated (Pietrangelo). Aggressive phlebotomy is a safe and effective treatment for all these disorders. All forms of genetic hemochromatosis are associated with decreased hepcidin production or activity (Papanikolaou et al., 2004, Roetto et al., 2003, Bridle et al., 2003, Nemeth et al., 2005). Considering this common feature, the diseases can be organized into three functional classes. First, mutations in the hepcidin gene prevent the production of active hepcidin protein, leading to unregulated ferroportin activity. Second, hemochromatosis-associated mutations in ferroportin prevent hepcidin binding and/or consequent ferroportin internalization and degradation, leading to unregulated ferroportin activity (De Domenico et al., 2006, Drakesmith et al., 2005).
The third class of mutations is in other genes—HfE, TfR2, and Hjv—that have been inferred to be necessary for appropriate hepcidin gene regulation. HFE, TFR2, and hemojuvelin are all expressed on the surface of hepatocytes. HJV acts as a BMP co-receptor. To date, the exact roles of HFE and TfR2 have not been reported, but it is reasonable to speculate that they, too, may participate in BMP signaling in some way (Andrews and Schmidt, 2007).

Iron overload can also be a secondary feature in patients with congenital anemias. Patients with thalassemia, due to mutations in the human globin genes, have ineffective erythropoiesis. As a compensatory response to attempt to increase red blood cell production, hepcidin levels in these patients fall (Kearney et al., 2007, Papanikolaou et al., 2005) leading to markedly increased intestinal iron absorption . In severe thalassemia syndromes, the anemia is treated with multiple red blood cell transfusions, thus adding to the iron burden. In contrast to patients with HH, who have normal erythropoiesis and can be treated by phlebotomy, in thalasemia treatment with iron chelator

agents is the only option to remove the excess iron (Andrews and Schmidt, 2007). Until recently, infusion of the natural product chelator, deferoxamine, was the standard of care, but new oral agents (deferiprone and deferasirox) are becoming important therapeutic options, particularly in patients who are poorly compliant with a daily infusion regimen (Viprakasit et al., 2009). Iron overload also occurs in patients lacking ceruloplasmin, the serum multicopper oxidase that oxidizes iron in concert with export from cells (Harris et al., 1998).

METABOLISM OF ALCOHOL (ETHANOL):

Ethanol is metabolized to acetate principally in the liver, with the generation of NADH. The principal route for metabolism of ethanol is through hepatic alcohol dehydrogenases, which oxidize ethanol to acetaldehyde in the cytosol. Acetaldehyde is further oxidized by acetaldehyde dehydrogenases to acetate, chiefly in mitochondria. Acetaldehyde, which is toxic, may enter the blood. NADH produced by these reactions is used for adenosine triphosphate (ATP) generation through oxidative phosphorylation. Most of the acetate enters the blood and is taken up by skeletal muscles and other tissues, where it is activated to acetyl CoA and is oxidized in the TCA cycle (Colleen Smith, 2005).

Approximately 10 to 20% of ingested ethanol is oxidized through a microsomal oxidizing system (MEOS), comprising cytochrome P450 enzymes in the endoplasmic reticulum (especially CYP2E1). CYP2E1 has a high Km (Michelis-Menten constant) for ethanol and is inducible by ethanol. Hence, the proportion of ethanol metabolized through this route is greater at high ethanol concentrations, particularly in chronic consumption of ethanol (Wu and Cederbaum, 2009).

Acute effects of alcohol ingestion arise principally from the generation of NADH, which greatly increases the $NADH/NAD^{-}$ ratio of the liver. As a consequence, fatty acid oxidation is inhibited, and ketogenesis may occur. The elevated $NADH/NAD^{--}$ ratio may also cause lactic acidosis and inhibit gluconeogenesis(Colleen Smith, 2005).

Ethanol metabolism may result in alchohol-induced liver disease, including hepatic steatosis (fatty liver), alcohol-induced hepatitis, and cirrhosis. The principal toxic products of ethanol metabolism include acetaldehyde and free radicals. Acetaldehyde forms adducts with proteins and other compounds. The hydroxyethyl radical produced by MEOS and other radicals produced during inflammation cause irreversible damage to the liver. Genetic polymorphisms in the enzymes of ethanol metabolism may be responsible for individual variations in the development of alcoholism or the development of liver cirrhosis (Colleen Smith, 2005).

ALCOHOL-INDUCED OXIDATIVE STRESS:

Multiple events are involved in the development of alcohol-induced oxidative stress in hepatocytes. These include changes in the redox state in cells, activation of cytochrome 2E1(CYP2E1) or NADPH oxidase and changes in mitochondrial functions.

Metabolism of alcohol, which is accompanied by oxidation and reduction reactions, causes an imbalance in the redox state of the cell by generating excess NADH (reduced nicotinamide adenine dinucleotide). CYP2E1 enzyme is induced in chronic alcoholism (Lu and Cederbaum, 2008). It is present in liver microsomes. Once induced, it continues to be metabolically active even in further absence of alcohol (Cederbaum, 2003). It produces reactive oxygen species, such as superoxide and hydrogen peroxide, leading to lipid peroxidation, oxidative stress and tissue injury. NADPH oxidase catalyzes the reduction of molecular oxygen to generate superoxide (Rotrosen et al., 1992, Chanock et al., 1994). The liver expresses both phagocytic and non-phagocytic iso-forms of NADPH oxidase (Reinehr et al., 2005) Alcohol-induced liver pathology was not observed in mice with deficient NADPH oxidase activity, suggesting a role for this enzyme in the progression of alcoholic liver disease (Kono et al., 2000).

Mitochondria play key roles in iron biogenesis and alcohol metabolism (Bailey and Cunningham, 2002, Aisen et al., 2001). Alcohol damages proteins of the mitochondrial electron transport chain

and decreases the rate of hepatic ATP synthesis (Bailey and Cunningham, 2002, Cunningham and Bailey, 2001). This induces the transfer of unpaired electrons to molecular oxygen and increases the production of reactive oxygen species.

ALCOHOLIC LIVER DISEASE:

Alcoholic liver disease comprises fatty liver, alcoholic hepatitis and finally cirrhosis. Mortality rates of approximately 60% have been encountered in alcoholic hepatitis with concomitant cirrhosis (Fauci, 2008). Alcohol by itself is hepatotoxic. The metabolism of alcohol also initiates a host of metabolic responses, such as formation of protein-aldehyde adducts, oxidative stress (Mantle and Preedy, 1999), immunologic activity (Willner and Reuben, 2005, Reuben, 2006) and release of pro-inflammatory cytokines (Reuben, 2006), All of these contribute to a hepatotoxic response. Several studies have shown that alcohol induces CYP2E1, a member of the P450 family showing high catalytic activity for ethanol. CYP2E1 causes hepatic injury by formation of various metabolites (Lu and Cederbaum, 2008, Bardag-Gorce et al., 2006, Kessova and Cederbaum, 2003, Cederbaum, 2006). It has been noted that a complex interaction between intestinal cells and hepatocytes is essential for alcohol-mediated liver injury (Kohgo et al., 2005). Intestine-derived endotoxemia and tumour necrosis factor-α (TNF-α) facilitate hepatocyte apoptosis and necrosis. They also activate stellate cells and fibrogenesis. It is the fibrogenesis which ultimately is responsible for the final hepatic architechture and loss of function in the disease process (Fauci, 2008).

ROLE OF IRON IN ALCOHOLIC LIVER DISEASE:

Alcohol consumption has been reported to cause alterations in iron homeostasis. These range from anemia to iron overload (Conrad and Barton, 1980, Friedman et al., 1988, Eichner, 1973, Whitfield et al., 2001). Causes for these vary. Alcoholic cirrhosis induces gastrointestinal blood loss (Kimber et al., 1965), which can lead to iron deficiency anemia. Nutritional deficiencies of folate are often seen in chronic alcoholics, resulting in megaloblastic anemia (Lindenbaum, 1980, Lindenbaum and Roman, 1980). Megaloblastic anemia, sideroblastic anemia and macrocytosis of alcoholism commonly occur in patients hospitalized for chronic alcoholism (Hines and Cowan, 1970, Sheehy

and Berman, 1960). Such iron deficiency can be prevented if adequate nutrition is maintained (Waters et al., 1966, Pierce et al., 1976). Moderate alcohol consumption (upto 2 alcoholic drinks per day) has also been shown to exert a protective effect by reducing the risk for iron deficiency anemia in adults. However, heavy drinking (more than 2 drinks per day) is associated with iron overload (Ioannou et al., 2004). A study in adolescents reveals that increased frequency of drinking increases serum iron concentrations, irrespective of gender. Adolescent males also showed high hemoglobin and serum transferrin saturation (Friedman et al., 1988). A positive correlation has been reported between alcohol consumption and ferritin levels in healthy adults, irrespective of gender (Milman and Kirchhoff, 1996). Bell et al. (Bell et al., 1994) have shown that elevated indices of iron associated with chronic alcoholic liver disease tend to normalize after 2-6 weeks of abstinence.

ROLE OF ALCOHOL IN HEPATIC IRON OVERLOAD:

Patients with alcoholic liver disease often display iron overload (Chapman et al., 1982, De Feo et al., 2001, Whitfield et al., 2001). Iron stores can be elevated even in mild to moderate of consumption of alcohol (Ioannou et al., 2004), as revealed by tissue iron staining reactions. Immuno-histochemical analysis of liver biopsy samples from patients of alcoholic liver disease (ALD) showed increased levels transferrin receptor-1 (Suzuki et al., 2002). Study of animal models have shown that both hepatocytes and Kuffper cells, which are a part of the reticulo-endothelial system (RES), show increased iron accumulation (Xiong et al., 2003). Alcohol and iron independently cause increased generation of free radicals, resulting in oxidative stress and lipid peroxidation (Pietrangelo, 1998, Bacon and Britton, 1990, Cederbaum, 2001, Feierman et al., 1985). Alcohol-induced iron overload in ALD induces an enhanced rate of production of pro-inflammatory cytokines which are also capable of producing hepatic injury (Valerio et al., 1996, Tsukamoto and Lu, 2001, Tsukamoto et al., 1995). These studies suggest that alcohol and iron-mediated intra-cellular signaling are inter-related.

Iron is absorbed maximally from the proximal small intestine; in vitro studies on duodenal biopsies from male alcoholics, using radioactive iron (^{59}Fe) demonstrated a 2-fold increase in intestinal iron

absorption (Duane et al., 1992). Alterations in intestinal permeability were also documented in these biopsy samples, using radioactive cyanocobalamine (^{57}Co) as an extracellular fluid marker. In view of this finding, it was concluded that the increased absorption of iron was through a para-cellular route (Duane et al., 1992).

The liver stores iron in both hepatocytes and Kupffer cells. Alcohol has the dual ability to either damage the hepatocytes by oxidative stress or cause iron overload by separate regulatory pathways. Several novel iron regulatory molecules have been discovered in recent years which can be modulated by alcohol (Harrison-Findik, 2007).

Effect of alcohol on molecules involved in iron homeostasis:

Alcohol is known to induce iron overload in the liver (Duane et al., 1992). Levels of iron regulate the synthesis of hepcidin , which plays a central role in iron metabolism (Pigeon et al., 2001, Nicolas et al., 2001). Harrison-Findik et al (2006) have shown, in vitro and in vivo, that exposure to alcohol down-regulates hepcidin production. Their in-vitro experiments, carried out on a hepatoma cell line, showed that the effect of alcohol on hepcidin expression in these cells was abolished by 4-methylpyrazole, a specific inhibitor of enzymes (both the alcohol and the aldehyde dehydrogenase) that metabolize alcohol. Their work on mice given alcohol (at concentrations of 10% and 20% in drinking water) for 1 week showed that alcohol did not alter the expression of transferrin receptor-1 and the iron storage protein, ferritin, or the activation of iron regulatory RNA-binding proteins, IRP1 and IRP2.

These results indicate that alcohol does not regulate hepcidin expression by altering the iron status of the cell but rather by acting on hepcidin directly. Short-term alcohol exposure was found to down-regulate hepcidin 1, but not hepcidin 2 mRNA expression in mice (Harrison-Findik et al., 2006), while iron has been shown to up-regulate hepcidin 1 gene expression in mice (Nicolas et al., 2002a).

Rats given alcohol for 12 weeks also displayed reduced hepcidin expression (Bridle et al., 2006, Harrison-Findik et al., 2007). These studies demonstrate that alcohol affects hepcidin expression in

the liver. Recent studies have revealed that alcohol-mediated oxidative stress down-regulated expression of hepcidin and caused increased expression of the duodenal iron transporters, DMT1 and ferroportin (Harrison-Findik et al., 2006). This effect was abolished by injection of hepcidin (Harrison-Findik et al., 2007). Increased ferroportin in the duodenum in mice with hemochromatosis mutations leads to increased absorption of iron in the blood, as revealed by increased serum iron levels in mice (Flanagan et al., 2007).

Iron is stored intracellularly as ferritin. There is evidence of increased levels of ferritin in rats given alcohol for 7 weeks (Harrison-Findik et al., 2007). This raises the question that if hepcidin down-regulation leads to iron accumulation, the resultant increase in the iron should cause hepcidin to be up-regulated to restore homeostasis. To answer this question, Harrison-Findik et al (2007) studied the combined effects of iron and alcohol on hepcidin levels. They showed that hepcidin levels were depressed in response to alcohol administration, despite a simultaneous iron overload. This study concluded that iron-mediated hepcidin regulation is compromised by alcohol.

Both alcohol and iron are known to produce oxidative stress-induced damage, which is thought to be important in the pathogenesis of ALD (Pietrangelo, 2003, Bacon and Britton, 1990, Cederbaum, 2001). Antioxidants have been shown to abolish the effect of alcohol on hepcidin expression in liver and duodenal transporters in the duodenum (Arteel, 2003, Cahill et al., 1997, Sun et al., 2001).

Effect of alcohol on Kupffer cells in the liver:

Moderate consumption of alcohol leads to iron deposition in hepatocytes. With increasing consumption of alcohol, iron begins to get deposited in Kupffer cells as well (Takada et al., 1993, Kohgo et al., 2005). Kupffer cells are a part of the reticuloendothelial system (RES). They take active part in iron metabolism by helping in recycling of iron (Knutson and Wessling-Resnick, 2003). Several studies were carried out to determine whether hepatocytes or Kupffer cells are involved in iron metabolism. A brief account of these studies is presented below.

It has been shown that expression of TrfR1 is increased in experimental animal models of and patients with ALD (Suzuki et al., 2002, Xiong et al., 2008) , compared with control animals or healthy human subjects, respectively. The increase in TrfR1 expression was observed in Kupffer cells of experimental animals while in patients with ALD, the increase was observed mainly in the hepatocytes (Suzuki et al., 2002, Xiong et al., 2008). Macrophages can take up transferrin-bound iron in vitro (Jacolot et al., 2008). However, a significant uptake of transferrin-bound iron by macrophages has not been observed in vivo in humans (Finch et al., 1970). It should also be noted that mice with disrupted TrfR1 gene, deficient in the global expression of TrfR1 receptor, display abnormalities only in erythropoiesis and neurologic development (Levy et al., 1999a).

Ethanol and its metabolites can damage the intestinal mucosa and cause lipopolysaccharides (LPS) derived from the resident gut bacteria to leak into the blood (Schaffert et al., 2009). This may account for alcoholics having raised levels of lipopolysaccharides in blood and liver. This behaves as an endotoxin and stimulates Kupffer cells to release pro-inflammatory cytokines, such as TNF-α, and reactive oxygen species (Tsukamoto et al., 2001, Wheeler, 2003). Studies have shown that the effect of alcohol on liver was blocked if the liver was depleted of Kupffer cells (Adachi et al., 1994, Koop et al., 1997). Kupffer cells of rats exposed to chronic alcohol have also been shown to display increased expression of Hfe, the gene for genetic hemochromatosis (Xiong et al., 2008). This HFE protein does not bind iron nor is it involved in direct uptake of iron but is thought to modulate hepcidin expression, leading to iron overload in the liver . Experiments have shown that Hfe knockout mice display an iron- overload phenotype (Levy et al., 1999b, Zhou et al., 1998). Studies in animal models have shown that increased iron levels in Kupffer cells led to increased expression of the transcription factor, nuclear factor-kappa beta (NF-kB), which led to increased production of pro-inflammatory cytokines, TNF-α and IL-6 (Tsukamoto et al., 1999, Xiong et al., 2003, She et al., 2002, Lin et al., 1997). These effects were abolished by chelation of iron (Lin et al., 1997).

Iron release from macrophages is also regulated by ferroportin (Andriopoulos and Pantopoulos, 2006, Knutson et al., 2003). Kupffer cells of animals exposed to alcohol have been shown to display

an increase in ferroportin expression, indicating a state of increased iron export (Xiong et al., 2008). As hepcidin leads to internalization and degradation of ferroportin, a decrease in hepcidin synthesis might be responsible for up-regulation of ferroportin (Harrison-Findik et al., 2007, Harrison-Findik et al., 2006, Ohtake et al., 2007).

Kupffer cells have not been shown to stimulate hepcidin synthesis in vivo (Montosi et al., 2005). (Theurl et al., 2008) showed that Kupffer cells have a role to play in a mouse model, where hepcidin production is up-regulated by inflammation but other studies have shown that depletion of Kupffer cells does not abrogate hepcidin up-regulation in the liver (Lou et al., 2005, Montosi et al., 2005).

The role of Kupffer cell activation and TNF-α signaling in the regulation of hepcidin expression by alcohol has been studied in vivo. Inactivation of Kupffer cells by gadolinium chloride in rats pair-fed with the Lieber DeCarli alcohol diet for 6 weeks or mice fed with ethanol in the drinking water for 1 week did not reverse alcohol-induced suppression of hepcidin (Harrison-Findik et al., 2009). When co-cultured with hepatocytes, Kupffer cells have been suggested to exert a negative effect on hepcidin synthesis in liver (Theurl et al., 2008). However, the depletion or inactivation of the Kupffer cells has been not been reported to induce any significant changes in basal hepcidin expression levels in the livers of control and alcohol-treated animals (Harrison-Findik et al., 2009). Harrison-Findik et al (2009) have also reported that a week of ethanol treatment (in the drinking water) was sufficient to induce NF-κB activation and TNF-α and IL-6 release in mice. . They also showed that neutralization of TNFα reduced NFkB production but neutralization of TNFα or suppression of TNFα I and II receptors did not down-regulate alcohol-mediated hepcidin production (Harrison-Findik et al., 2009). All these studies suggest that Kupffer cells may not be involved in liver hepcidin expression in vivo.

Effect of alcohol on hepcidin production in hepatocytes:

Alcohol-induced oxidative stress has been shown to be involved in the suppression of hepcidin promoter activity and hepcidin transcription in the liver *in vivo* in mice, by inhibiting the DNA binding

activity of the transcription factor, C/EBPα (Harrison-Findik et al., 2006). C/EBPα (also known as CEBPA) is a transcription factor that binds as a homodimer to promoter sequences of a variety of genes (Andrews and Schmidt, 2007). Thereby hepatocytes assume a predominant role in the regulation of hepcidin expression and iron metabolism by alcohol.

Several factors including iron, viral infections, endotoxins and reactive oxygen species have been implicated to act as second-hit factors in the progression of alcoholic liver disease. It is known that hepcidin expression in the liver is regulated by a multitude of factors such as iron, alcohol, hepatitis C viral proteins, inflammation, hypoxia and oxidative stress (Harrison-Findik et al., 2009, Nemeth et al., 2004a, Lee et al., 2005, Nishina et al., 2008). Nishina et al (Nishina et al., 2008) have recently shown that hepatitis C viral protein-mediated oxidative stress suppresses hepcidin transcription by altering the activity of the transcription factor, C/EBP α. Together, these findings demonstrate a role for alcohol-mediated oxidative stress in the inhibition of C/EBP α activity and reduced hepcidin transcription(Harrison-Findik et al., 2007, Harrison-Findik et al., 2006). Thus, reduced hepcidin expression results in iron deposition in hepatocytes which may act as a second hit in the progression of ALD.

THE

STUDY

HYPOTHESIS OF THE STUDY

Alcohol ingestion increases the expression and function of molecules that are involved in the absorption of non-haem iron from the gastrointestinal tract, thereby leading to iron overload.

OBJECTIVES OF THE STUDY

The aim of this study was to determine the effect of alcohol ingestion on proteins in the mouse duodenum that are involved in transport of non-haem iron from the intestinal lumen into the systemic circulation. This was to be done by determining expression levels of 4 proteins of interest, namely, divalent metal transporter1 (DMT1), duodenal cytochrome reductase (dcytb), ferroportin and hephaestin.

MATERIALS

AND

METHODOLOGY

MATERIALS

Equipment used:

1. Elix and Milli-Q ultrapure water systems (Millipore, USA)
2. pH meter (Systronics, India)
3. Refrigerated centrifuge (MPW R 350, MPW Poland)
4. Glass homogenizer and teflon pestle (1 ml capacity) (Kimble-Kontes, USA)
5. Mechanical homogenizer (REMI, Bombay, India)
6. Apparatus for SDS-PAGE and western blotting (Bio-Rad, USA)
7. Gel documentation system (Alpha Innotech, USA).
8. Real time thermocycler (Chromo4, Biorad, USA)

Chemicals and reagents used:

1. Acrylamide, ammonium persulfate, β-actin antibody, β-mercaptoethanol, bovine serum albumin (BSA), chloroform, diethyl pyrocarbonate (DEPC), ethidium bromide, ferric chloride hexahydrate, guanidine hydrochloride, isopropanol, sodium lauryl sulphate (SDS) and TRI reagent were obtained from Sigma, India.
2. Casein (SRL, India)
3. Disodium hydrogen phosphate, potassium chloride, potassium dihydrogen phosphate and sodium acetate were obtained from Sisco Research Laboratories, Pvt Ltd, Mumbai, India.
4. Absolute alcohol (99.9%) (Hayman, England).
5. Agarose (Genei, Bangalore, India)
6. 3 – Morpholinopropane sulfonic acid (MOPS) (Fluka Biochemika, Sigma, India)
7. Reverse transcription core kit, SYBR green PCR master mix kit and gene-specific primers were obtained from Eurogentec, Belgium.
8. Methanol (Qualigens)
9. Skimmed milk powder (Sagar, India)
10. Anti-DMT1 antibody (Santa Cruz, USA)
11. Anti-ferroportin antibody (Alpha Diagnostics International,USA)

12. Goat anti-mouse and anti-rabbit peroxidase-conjugated antibodies and chemiluminescent substrate were purchased from Pierce International, USA.

All chemicals used were of analytical grade.

Diet used for the experimental mice:

1. Lieber-DeCarli alcohol and control diets (Dyets Incorporated, USA).

Anaesthetic agent used when handling the mice:

1. Halothane (Nicholas Piramal India, Ltd).

METHODOLOGY

Animals used:

Swiss albino male mice (weighing around 30 g), obtained from the animal house, Christian Medical College, Vellore, were used for all the experiments. They were divided into pairs of age and weight-matched control and test animals. The pairs were allotted for study periods of 1, 2 and 4 weeks. The experiments done were approved by Institutional Review Board (Approval No. 6589 dated 11th June, 2008) and the Institutional Animal Ethics Committee (IAEC) and were in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA), Government of India (Approval No.7/2009 dated 27th May, 2009).

Preparation of Lieber-DeCarli control and ethanol diets:

The Lieber-DeCarli control and ethanol diets were prepared as follows:

Diet given	Lieber-DeCarli diet powder (Dyets) (g)	Casein (g)	Maltose-dextrin (g)	95% ethanol (ml)
Lieber-DeCarli (Control)	90.78	41.4	92.11	Not applicable
Lieber-DeCarli (Ethanol)	90.78	41.4	39.47	37.38

The amount of each constituent used was as recommended by the manufacturers. The constituents were blended in cold water for 30 seconds and the final volume made up to 1 litre.

Administration of the Lieber-DeCarli diets:

Mice were placed in individual cages and pair-fed for the periods of the study with the Lieber-DeCarli ethanol or control diets. The diet was placed in graduated bottles specially purchased for the purpose. Mice that were given ethanol were allowed to drink from these *ad libitum*. The volume of diet consumed over 24 hours by each alcohol-treated mouse was recorded each day. The control mouse in each pair was provided with the same volume of control diet over the next 24 hours, thus

achieving pair-feeding. This ensured that ethanol-treated and control mice received equivalent number of calories in their diets. Thus, differences in calorie intake were eliminated as a

confounding factor in these experiments. Total calories in the control diet were provided by maltose-dextrin in the control diet. In the Lieber-DeCarli ethanol diet used in this study, 20% of the total calories were replaced by ethanol (100%). The amount of alcohol to be added to achieve this was calculated using a conversion table provided by the manufacturers.

One litre of the Lieber-DeCarli ethanol diet contained 37.38 ml alcohol (table 1). The amount of alcohol consumed per day by each mouse was calculated on the basis of the following formula:

Amount of alcohol consumed by each mouse per day = volume of Lieber-DiCarli (ethanol diet) consumed (ml) per 24 hours x (37.38/1000)

Documentation of changes in weights of mice:

The weight of each mouse was recorded prior to its allocation into a time period (day 1) and before its sacrifice at the end of the specified time period (i.e weights were checked on day 7 for the 1 week group, day 14 for the 2 weeks group and day 28 for the 4 weeks group).

Sacrifice of mice:

At the end of each period of study (day 8 for the 1 week group, day 15 for the 2 weeks group and day 29 for the 4 weeks group), the mice were sacrificed by cervical vertebral dislocation under halothane anaesthesia. A laparotomy was done and the duodenum was isolated. The mucosal surface of the duodenum was scraped, using a glass slide. The scrapings were snap-frozen in liquid nitrogen and stored at -70^{o}C till further processing.

RNA ISOLATION, QUANTITATION AND STORAGE:

Isolation of RNA:

Total RNA was extracted from the mucosal scrapings, as described below:

Step 1: To each 50-100 mg of tissue, 1 ml of TRI reagent (Sigma) was added. The samples were then homogenized in a glass homogenizer, using a mechanical homogenizer (25-30 strokes).

Step 2: Each homogenized sample was kept on ice for 10 minutes. To this, 200 μl of chloroform was added. The mixture was shaken vigorously for 30 seconds. It was then placed on ice for a further period of 15 minutes.

Step 3: The mixture was centrifuged at 12000*g* for 15 minutes at 4^{o}C. At the end of the centrifugation, three layers had formed: an upper aqueous layer, an inter-phase and a lower organic layer. The upper aqueous layer contained RNA while the inter-phase contained genomic DNA. The lower organic (phenol) layer was composed of cellular protein and debris.

The upper aqueous phase was used for isolation of total RNA. The middle inter-phase and the lower organic layer were preserved in a micro-tube at -70^{o}C for protein isolation from the samples.

Step 4: About 500 µl of the upper aqueous layer was transferred to a fresh 1.5 ml micro-tube. Care was taken to not disturb the interface in order to avoid DNA contamination.

Step 5: To the aqueous portion that was removed, 500 µl of pure isopropanol was added. The mixture was allowed to stand on ice for 10 minutes.

Step 6: The mixture was then centrifuged at 12000*g* for 10 min at 4^{o}C.

Step 7: The supernatant obtained was discarded and 1ml of 75% ethanol in water was added to thetube. The contents of the tube were gently mixed, using a vortex mixer.

Step 8: The mixture was then centrifuged at 12000*g* for 5 min at 4^{o}C.

Step 9: The supernatant was discarded and the RNA pellet obtained was air-dried.

Step 10: The dried pellet was dissolved in 10-20 µl of DEPC water, by placing the tube in a heated water bath (55-65^{o}C).

Assessment of integrity of RNA obtained:

The integrity of the RNA isolated was checked by electrophoresing an aliquot of the sample in a 1.2% formaldehyde-agarose gel. The presence of 2 distinct bands, representing the 28S and 18S ribosomal subunits of RNA, in an approximate band density ratio of 2:1, was considered evidence of intact RNA.

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Quantitation of isolated RNA:

Isolated RNA was quantified by spectrophotometry.

Principle: Nucleic acids strongly absorb ultraviolet light at a wavelength of 260nm.

An optical density reading of 1.0 at 260 nm is taken to indicate an RNA concentration of 40 µg/ml.

Procedure: Each RNA sample was quantitated against a DEPC water blank using a 'Nanodrop' spectrophotometer. 1 µl of DEPC water was used as a blank and then 1µl each of the samples were loaded on the pedestal of the spectrophotometer set at a measuring wavelength of 260 nm. The pedestal was carefully cleaned with a tissue paper before the succeeding sample was loaded. The spectrophotometer software automatically calculated the RNA values using the formula mentioned above.

Storage of isolated RNA:

The final solution containing RNA in DEPC water was divided into aliquots and stored at -70^{o}C until further analysis.

Precautions observed during RNA isolation:

1. All materials used during the process of isolation (such as micro-tubes, water and reagents) were autoclaved prior to use. Commercially available sterile hand gloves were used throughout the procedures.

2. All pipetting procedures were carried out in a clean hood.

3. The tissues obtained after sacrifice of the animals were immediately snap-frozen, to prevent RNA degradation and stored at -70^{o}C. Care was taken to see that the tissue was completely homogenized.

4. The aqueous phase obtained was removed carefully without disturbing the intermediate layer containing DNA and the lower phase containing protein.

5. Care was taken to see that the final RNA pellet was completely dissolved in sterile DEPC water.

REVERSE TRANSCRIPTION AND POLYMERASE CHAIN REACTION (RT-PCR):

cDNA construction:

cDNA was synthesized using the reverse transcriptase core kit purchased from Eurogentec, according to the manufacturer's instructions. For this, 1µg of total RNA was used for reverse transcription to cDNA. The components of a single (10µl) reverse transcription reaction were as follows:

a) 10X reaction buffer – 1 µl (final concentration – 1X)

b) 25 mM $MgCl_2$ - 2 µl (final concentration – 5 mM)

c) 2.5 mM dNTP - 2 µl (final concentration – 500 µM)

d) Random nonamers – 0.5 µl (final concentration –2.5µM)

e) RNase inhibitors – 0.2 µl (0.4 U/µl)

f) Reverse transcriptase - 0.25 µl (1.25 U/µl)

g) RNase free water – to make final volume up to 10µl after addition of template.

h) Template (1 µg of total RNA)

A master mix was prepared using all the components of the reaction mixture except RNase-free water and the RNA template. Appropriate volumes of the master mix were then added to each PCR tube. The template was added next to each tube. Finally, RNase-free water was added to make the final volume of the reaction mixture to 10µl. Negative control tubes were also used for the reactions. These consisted of a tube which contained all the above reagents except reverse transcriptase and another which had all the reagents except the RNA template. The latter control was to eliminate the possibility of the RNA sample being contaminated with genomic DNA. The tubes were subjected to

centrifugation briefly in a microfuge. They were then placed in the real-time thermocycler (Chromo4, Bio-Rad). The reverse transcription reaction was programmed as follows:

Initial step: 10 min at 25^{o}C

Reverse transcription step: 30 min at 48^{o}C

Inactivation of reverse trancriptase: 5 min at 95^{o}C

At the end of the run, the samples were stored at -20 ^{o}C until further analysis.

Real-time polymerase chain reaction assays:

The cDNA that was obtained by reverse transcription was subject to amplification by the polymerase chain reaction (PCR). The components for performing a single 25 µl real-time PCR reaction were as follows:

1) cDNA template – 2.5 µl of the reaction mixture obtained at the end of reverse transcription

2) SYBR green Mastermix 2X – 12.5 µl

3) Gene-specific primers – 5 µl

4) Autoclaved ultrapure water - 5 µl

The reactions were set up in a 96-well plate format and all samples were analysed in triplicate to ensure reproducibility.

Gene-specific primers used for the PCR assays for the genes of interest are shown below:

Mouse DMT-1 IRE (Takeuchi et al., 2005)

Forward primer: 5´-CTG CTG AGC GAA GAT ACC-3´

Reverse primer: 5´-GTA AAC CAT AGA AAC ACA CTG G-3´

Mouse dcytb (Dupic et al., 2002)

Forward primer: 5´-GCAGCGGGCTCGAGTTTA-3´

Reverse primer: 5´-TTCCAGGTCCATGGCAGTCT-3´

Mouse ferroportin (Dupic et al, 2002)

Forward primer: 5´-TTGCAGGAGTCATTGCTGCTA-3´

Reverse primer: 5´-TGGAGTTCTGCACACCATTGAT-3´

Mouse hephaestin (Dupic et al, 2002)

Forward primer: 5´-TTGTCTCATGAAGAACATTACAGCAC-3´

Reverse primer: 5´-CATATGGCAATCAAAGCAGAAGA-3´

Mouse beta-actin (Dupic et al, 2002)

Forward primer: 5´-GACGGCCAAGTCATCACTATTG-3´

Reverse primer: 5´-CCACAGGATTCCATACCCAAGA-3´

The annealing and extension temperatures and times for each gene of interest and the concentrations of primers used were optimized for all the five genes studied (Table 2). The optimal salt ($MgCl_2$) concentration for each reaction was determined to be 5 mmol/L.

Validations of the reaction conditions were done using a standard curve, generated with serial dilutions of the cDNA template for each gene. The reactions were performed in areal-time thermocycler, programmed using Opticon Monitor software as follows:

1. Incubation at 50^{o}C for 2 min
2. Incubation at 95^{o}C for 10 min
3. Denaturation step at 95^{o}C for 1 min
4. Annealing step was done at the temperature and time optimized for each gene, as shown in table 2.
5. Extension step – 72^{o}C for 1 min
6. Readings were taken
7. Step 3 onwards was repeated for 39 more cycles
8. Melting curve analysis was done from 60 to 95^{o}C, read at every 1^{o}C.

9. Samples were then cooled and maintained at 4^{o}C within the cycler.

10. End

The products obtained were stored at – 20^{o}C.

Table 2:

Primer	Annealing temp & time	Extension temp & time	Concentration of forward (F.P) and reverse primers (R.P.)
Beta-actin	58^{O}C X 20 secs	72^{O}C X 40 secs	F.P = 0. 828 µmol/L R.P = 0. 828 µmol/L
DMT-1	52.6^{O}C X 20 secs	60^{O}C X 40 secs	F.P = 0. 120 µmol/L R.P = 0. 125 µmol/L
Dcytb	60^{O}C X 20 secs	72^{O}C X 1 minute and 40 secs	F.P = 0. 540 µmol/L R.P = 0. 566 µmol/L
Ferroportin	62^{O}C X 20 secs	72^{O}C X 40 secs	F.P = 0. 276 µmol/L R.P = 0. 276 µmol/L
Hephaestin	59.5^{O}C X 20 secs	72^{O}C X 40 secs	F.P = 0. 268 µmol/L R.P = 0. 276 µmol/L

ANALYSIS OF DATA OBTAINED FROM THE PCR ASSAYS:

Background fluorescence was normalized by setting the baseline at zero. The outcome parameter generated at the end of the PCR was the cycle threshold (Ct) value. The term C_t or cycle threshold is defined as the PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold (Nolan et al. 2006). The threshold was determined by examining the amplification plot on a log-linear scale. The point where the cycles are parallel for all samples was considered to be an appropriate setting point for the threshold.

The efficiency of the PCR for each gene was determined by the following equation:

m = -(1/logE) where m is the slope of the standard curve and E is the efficiency.

DETERMINATION OF EXPRESSION LEVELS OF GENES OF INTEREST:

The expression levels of the genes of interest were calculated relative to those of an internal reference gene. This is a procedure that is done commonly in qRT-PCR assays to control for errors. The internal reference gene is presumed to not change under experimental conditions used in the system and hence can control for errors while performing a qPCR and hence minimizes variability between samples. The reference gene used in this study was β-actin. Data for each gene of interest was normalized relative to those for beta-actin. This was done by subtracting the Ct values of beta-actin in each sample from that of the gene of interest eg, ferroportin. The value obtained was referred to as the delta Ct (ΔCt) value.

Delta Ct (ΔCt) = (Ct of the gene of interest) – (Ct of beta-actin)

The relative fold-change in the gene of interest was determined according to the comparative Ct method, using the following formula:

Relative fold-change in gene of interest = $2^{-\Delta Ct}$ (Schmittgen and Livak, 2008)

PROTEIN ISOLATION AND QUANTITATION:

Isolation of proteins:

The following procedure was used to isolate proteins from the organic (phenol) phase obtained after centrifugation of the duodenal homogenate (prepared in TRI reagent).

Step1: Removal of DNA:

Ethanol (0.3ml of 100% ethanol) was added to the inter-phase and phenol layers obtained. This caused precipitation of DNA. Samples were kept at room temperature for 3 minutes. They were centrifuged at 2500*g* at 4^{o}C for 5 minutes. The DNA pellet obtained was discarded. The phenol-ethanol supernatant was preserved for protein isolation.

Step2: Precipitation of proteins:

Proteins were precipitated by adding 600μl of acetone to 200μl of the phenol- ethanol supernatant. The samples were kept at room temperature for 10 minutes. They were then centrifuged at 12,000*g* at 4^{o}C for 10 minutes.

Step3: The pellet obtained was dispersed with a teflon pestle in 500μl of a wash solution (0.3M guanidine hydrochloride in 95% ethanol and 2.5% glycerol [v/v]). An additional 500μl of the wash solution was then added to the sample and kept at room temperature for 10 minutes. The sample was then centrifuged at 8000*g* for 5 minutes. The wash solution was then removed by decantation. The pellet was washed twice more in 1 ml of the wash solution, as described above, to remove any residual phenol. The pellet obtained was subjected to a final wash in 1ml of ethanol containing 2.5% glycerol (v/v). At the end of 10 minutes of standing in the ethanol wash, the sample was centrifuged at 8000*g* for 5 minutes at 4^{o}C to obtain a protein pellet. The ethanol layer was decanted and the pellet dried at room temperature for 10 minutes.

Step4: The pellet obtained was finally dissolved in 200 μl of a 1% SDS solution. This was used for protein estimation and for western blots as described below:

Estimation of content of protein:

Protein was estimated by Lowry's method (Lowry et al., 1951). This method involves pre-treatment of protein with alkaline copper sulphate in the presence of tartrate, followed by addition of Folin's phenol reagent (a mixtue of phosphomolybdic-tungstic acids). The peptide bonds reduce the cupric ions in an alkaline medium to cuprous ions which then react with the Folin's phenol reagent. The phospohomolybdotungstate in Folin's phenol reagent is reduced to heteropolymolybdenum blue by copper-catalyzed oxidation of aromatic amino acids, to give rise to a blue coloured solution. The intensity of the colour depends on the tyrosine and tryptophan content of the protein. It is measured spectrophotometrically at 650nm.

Reagents:

1. Lowry's reagent
2. Folin's reagent (diluted 1:1 with deionized water).

Assay: Protein solutions obtained were diluted 1:10. From each sample, 25 and 50 μl were each added to 2.5 ml of freshly prepared Lowry's reagent. The total volume in each tube was made up to 3 ml with deionized water. The tubes were incubated at room temperature for 10 minutes. Folin's phenol reagent (0.25 ml) was added to each tube. The reaction mixture was incubated at room

temperature for 30 minutes. The blue colour produced was measured using a spectrophotometer, at a wavelength of 660 nm.

WESTERN BLOTTING:

Proteins (100μg) were denatured in protein dissociation buffer containing 62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 0.004% bromophenol blue and 5% β-mercaptoethanol for 5 minutes at 95^{o}C, separated on 10% polyacrylamide gels at 80V and electroblotted on to polyvinylidene difluoride (PVDF) membranes by overnight transfer at 25V. A commercially available molecular weight marker was also separated on the 10% polyacrylamide gel and electroblotted onto the PVDF membrane along with the samples. The membranes were then incubated in a blocking buffer (5% milk powder and 0.02% Tween-20 in PBS at pH 7.4) for 2 hours at room temperature. This was followed by incubation overnight with anti-DMT1 primary antibody (dilution 1 in 200 in PBS with 5% milk powder and 0.02% Tween-20) or anti-ferroportin antibody (dilution 1 in 400 in PBS with 5% milk powder and 0.02% Tween-20) at 4^{o}C in a cold room. The membranes were washed 3 times for 10 minutes each, using PBS with 0.02% Tween 20 at pH 7.4. They were then incubated in goat anti-rabbit secondary antibody and a commercially available S protein-HRP conjugate [required for visualization of the molecular weight markers](dilution 1 in 1000 and 1 in 2500 in PBS with 5% milk powder and Tween-20 respectively) for 2 hours at room temperature, The secondary antibody and the S protein was tagged with a peroxidase that acted on a chemiluminescent substrate. The bands seen were digitized using a CCD frame digitizer (Alpha Innotech) and quantified by densitometry, on a gel documentation system (Alpha Innotech), using AlphaEase software (Alpha Innotech).

The membrane was then treated with stripping buffer (100mM beta-mercaptoethanol, 2% SDS and 62.5mM Tris-HCl) for 30 minutes at 60^{o}C to remove the primary and secondary antibodies. It was then washed thrice in ultrapure water for 10 minutes each time. Final washes were done twice with a wash buffer (PBS with 0.02% Tween 20, at pH 7.4) for 15 minutes each time. The membrane was then incubated with antibody against beta-actin (dilution of 1:5000) overnight at 25V at 4^{o}C. The membrane was then washed 3 times for 10 minutes each and incubated in goat anti-mouse

secondary antibody (dilution 1 in 5000 in PBS containing 5% milk powder and 0.02% Tween 20). The bands were visualized and quantified as described above.

MEASUREMENT OF NON-HAEM IRON IN SERUM:

A sample of serum (20µl) was incubated with 20µl of acid reagent (3M hydrochloric acid and10% trichloroacetic acid) for 5 min. The mixture was then centrifuged at 3000*g* for 2 minutes. A clear supernatant was obtained along with a pellet in the microfuge tube. Two microlitres of this supernatant were mixed with 100µl of chromogen reagent (7M sodium acetate, 0.01% bathophenanthroline disulphonic acid and 0.1% thioglycollic acid). Absorbance of the resultant mixture was measured at 540 nm in a microplate reader. Serial 1:2 dilutions of a stock solution of ferric chloride hexahydrate (10g/L) in 0.1N HCl was used to prepare a total of 7 standards. The concentrations of these standards were 500µg/ml, 250µg/ml, 125µg/ml, 62.5µg/ml, 31.25µg/ml, 15.625µg/ml and 7.8125µg/ml respectively. A calibration curve was prepared each time the samples were assayed.

STATISTICAL ANALYSIS:

Statistical analysis of data obtained was carried out using Excel, Microsoft Corporation, USA and SPSS, SPSS Inc., USA, Version 16. The unpaired t-test was used to compare means of data from the alcohol-treated and corresponding control mice. One way ANOVA was used to compare the means of data from mice at the different periods of study. A p-value of less than 0.05 was taken to indicate statistical significance in all cases.

RESULTS

1. TEST FOR NORMAL DISTRIBUTION OF VALUES

All variables in this study were found to have a normal distribution as assessed by the one sample Kolmogorov-Smirnov test.

2. BODY WEIGHTS OF CONTROL AND ETHANOL-TREATED MICE

There were no significant differences in body weights of the ethanol-treated and control animals (Figure 1).

Figure 1

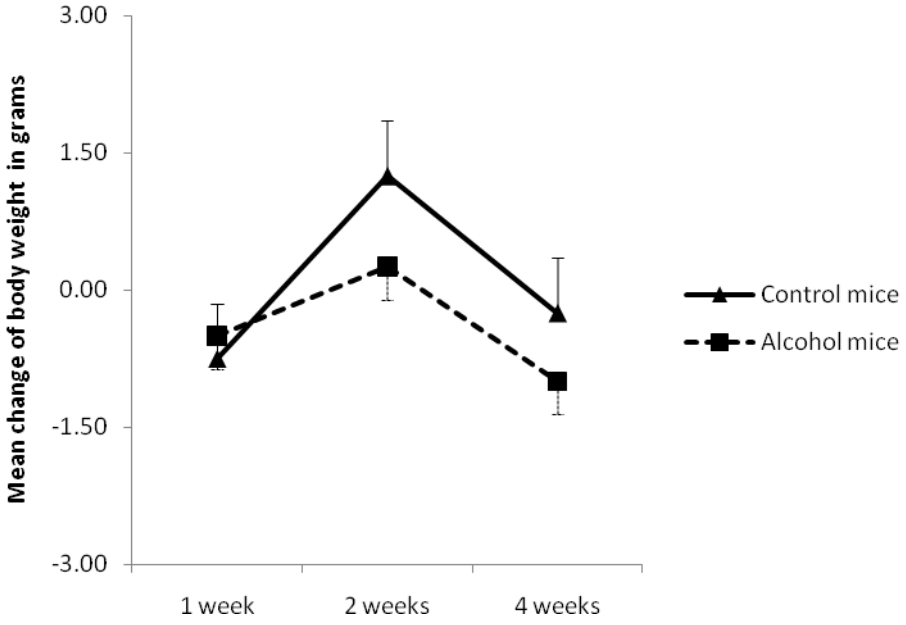

Figure1. Changes in body weight in control and alcohol-treated mice at the different time periods studied (N = 4 in each time period). Data shown are mean ± S.E. in each group.

3. ALCOHOL CONSUMPTION IN THE ETHANOL-TREATED MICE OVER VARIOUS TIME PERIODS

There were no significant differences seen in the amount of ethanol consumed by the mice over the various time periods studied (i.e. 1 week, 2 weeks and 4 weeks) (Figures 2a and 2b).

Figure 2a

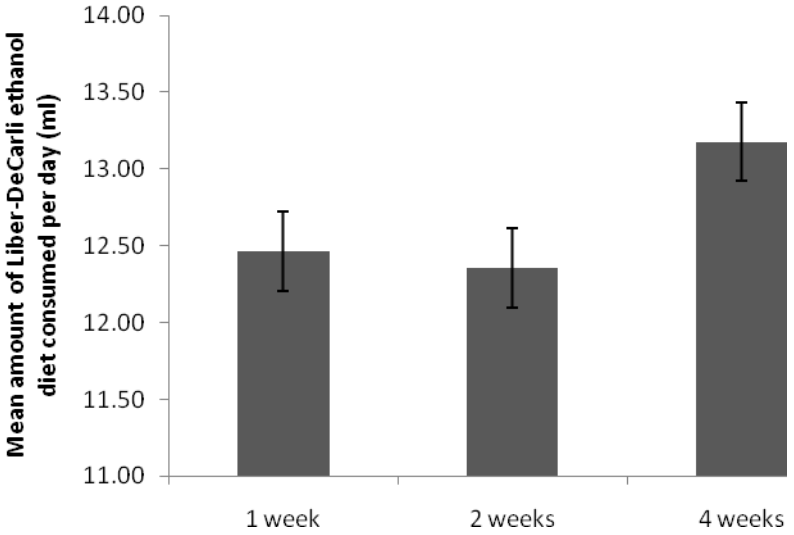

Figure 2a. Amount of alcohol consumed by mice per day in each experimental group (N = 4 in each time period). The data represents mean ± S.E. in each group.

Figure 2b

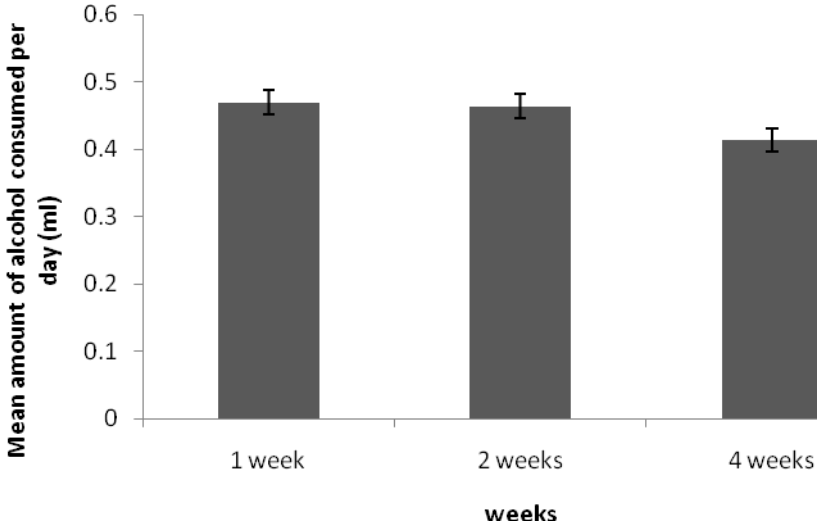

Figure 2b. Amount of alcohol consumed by alcohol-treated mice per day in each experimental group (N = 4 in each time period). The data represents mean ± S.E. in each group.

4. OPTIMIZATION OF PCR ASSAYS FOR TARGET GENES BY GENERATION OF STANDARD CURVES AND MELT CURVE ANALYSES

The log fluorescence data graph, standard curves and melting curves generated for each gene of interest were obtained directly from the Opticon Monitor software used, after completion of the PCR assay for each gene. The efficiencies of the PCR for all the genes studied were found to be similar.

a) DMT1:

Figures 3a, b and c show the log fluorescence data, standard curve and melting curves respectively for DMT-1. The average efficiency of the PCR for DMT-1 was found to be 100%. The melting curve analysis showed the presence of a single peak at 78-79^{o}C (Fig 3a, b and c).

Figure 3a.

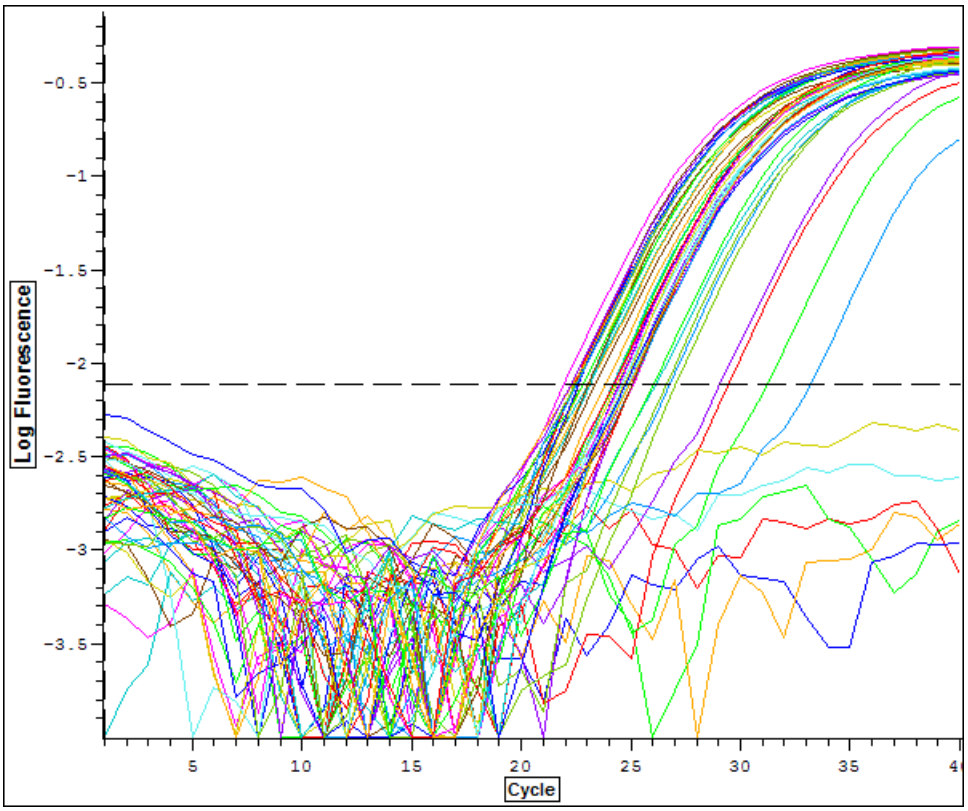

Figure 3a. Log fluorescence data graph for DMT-1. The graph depicts the PCR cycle number on the x-axis and relative log fluorescence units on the Y axis. The dotted line shows the cycle threshold is set at a point above baseline fluorescence.

Figure 3b.

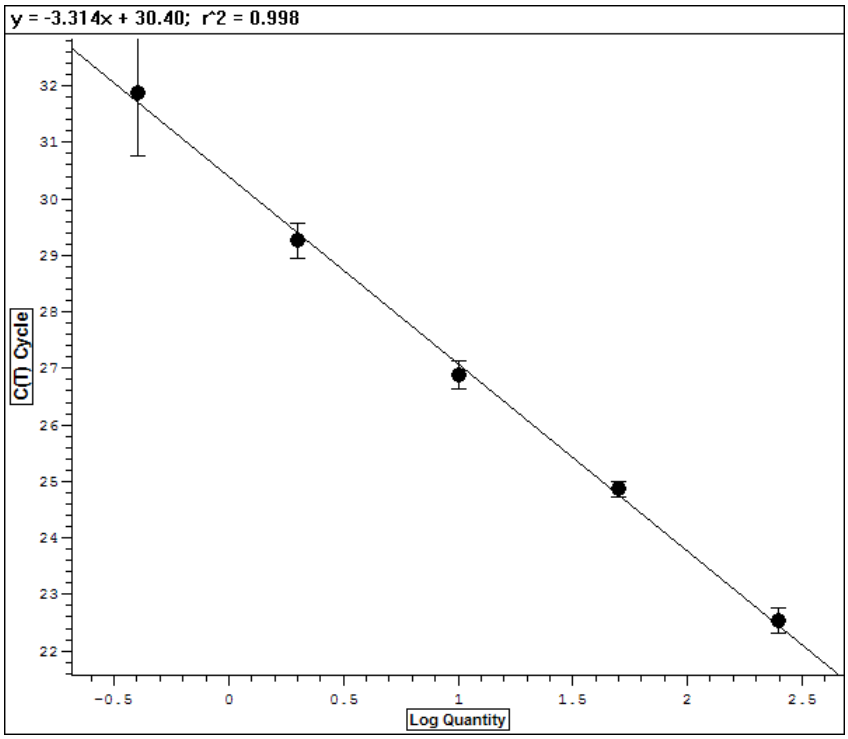

Figure 3b. Standard curve for DMT-1. The graph depicts log of the quantity of cDNA on the x-axis and cycle threshold value on the y-axis for each of the standard samples used.

Figure 3c.

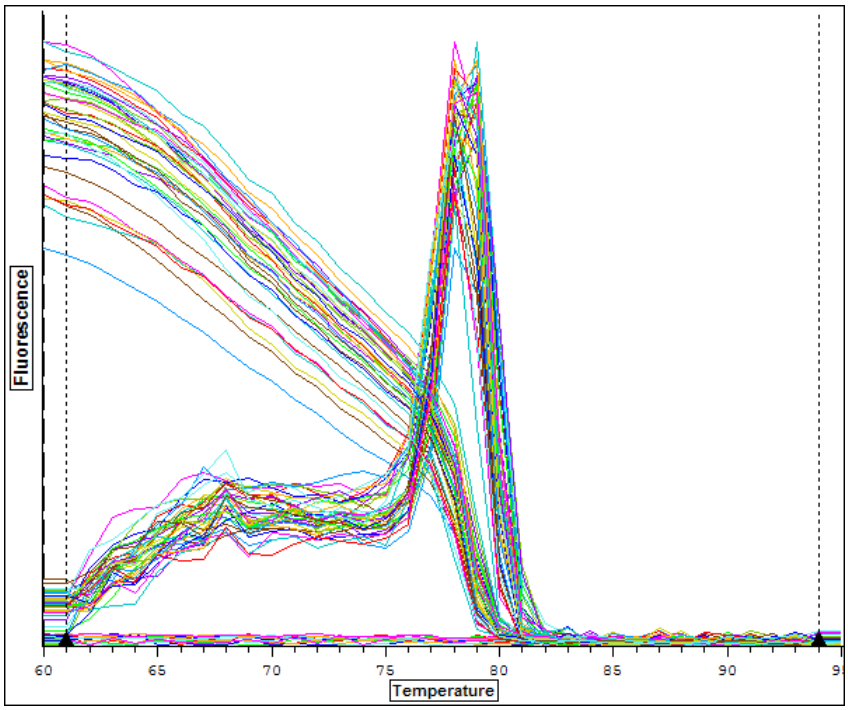

Figure 3c. Melting curve for DMT-1. The graphical plot of the melting curve was obtained after the PCR, using DMT-1-specific primers. The presence of a single peak at 79-80^{o}C is suggestive of the generation of a specific product during the PCR.

b) Ferroportin:

Figures 4a, b and c show the log fluorescence data, standard curve and melting curves respectively for ferroportin. The average efficiency of the PCR for ferroportin was found to be 101%. The melting curve analysis showed the presence of a single peak at 82-83^{o}C.

Figure 4a

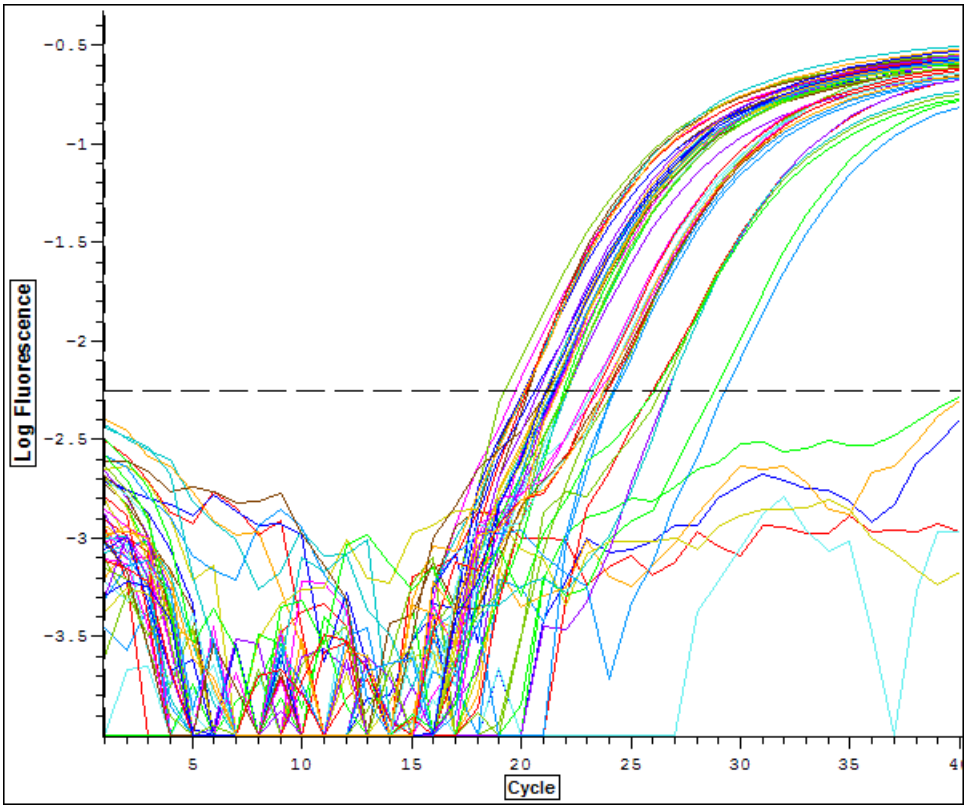

Figure 4a. Log fluorescence data graph for ferroportin. The graph depicts the PCR cycle number on the x-axis and relative log fluorescence units on the Y axis. The dotted line shows the cycle threshold is set at a point above baseline fluorescence.

Figure 4b

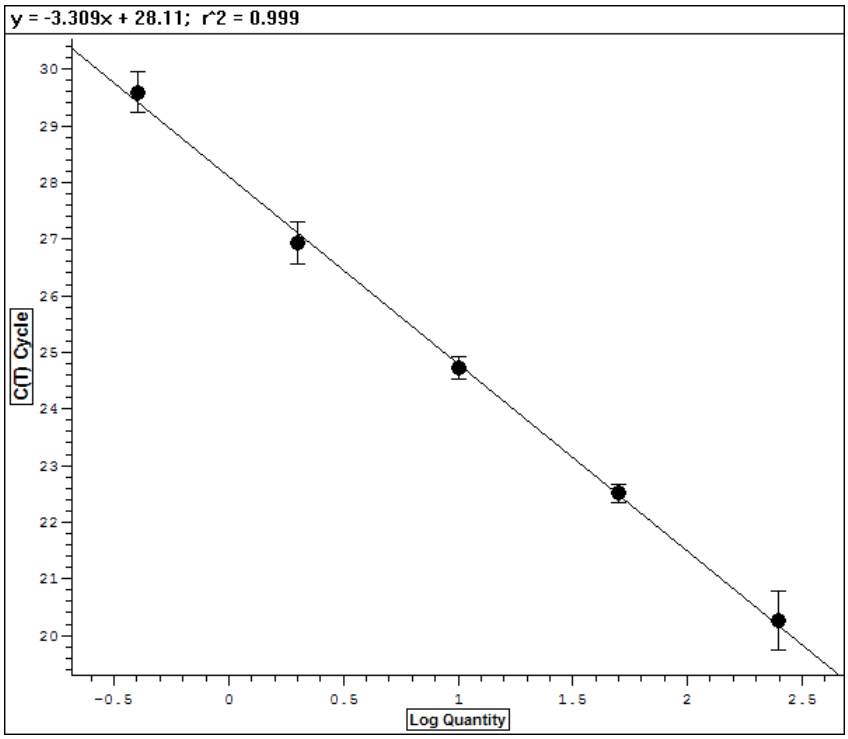

Figure 4b. Standard curve for ferroportin. The graph depicts log of the quantity of cDNA on the x-axis and cycle threshold value on the y-axis for each of the standard samples used.

Figure 4c

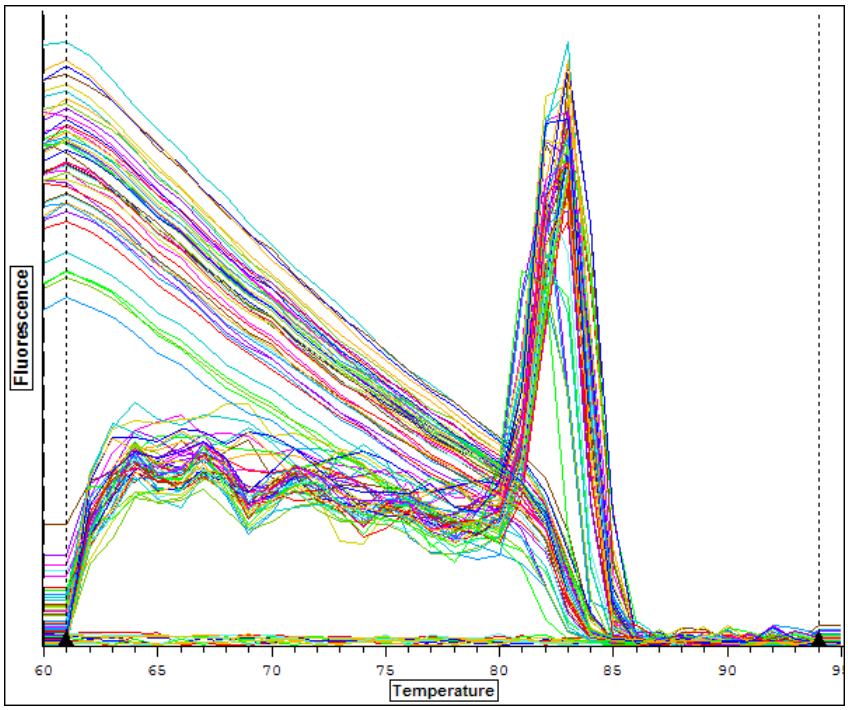

Figure 4c. Melting curve for ferroportin. The graphical plot of the melting curve was obtainedafter the PCR, using ferroportin-specific primers. The presence of a single peak at 81-82^{o}C is suggestive of the generation of a specific product during PCR.

c) Duodenal cytochrome b (dcytb):

Figures 5a, b and c show the log fluorescence data, standard curve and melting curves respectively for dcytb. The average efficiency of the PCR for dcytb was found to be 98%. The melting curve analysis showed the presence of a single peak at 82-83^{o}C.

Figure 5a

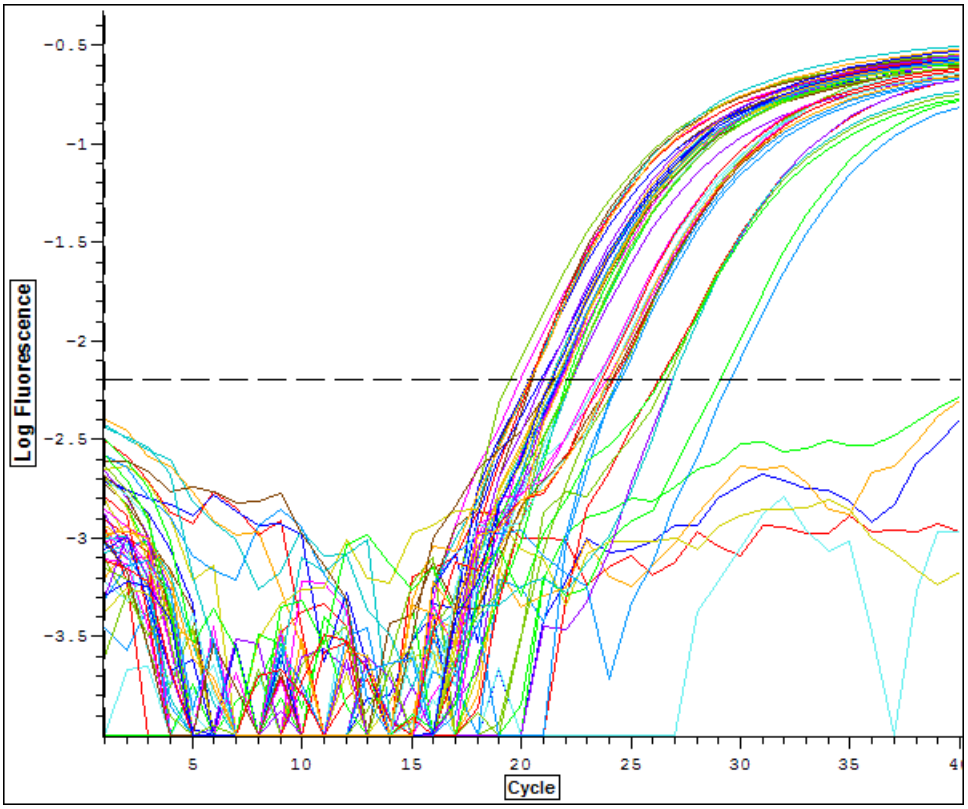

Figure 5a. Log fluorescence data graph for dcytb. The graph depicts the PCR cycle number on the x-axis and relative log fluorescence units on the Y axis. The dotted line shows the cycle threshold is set at a point above baseline fluorescence.

Figure 5b

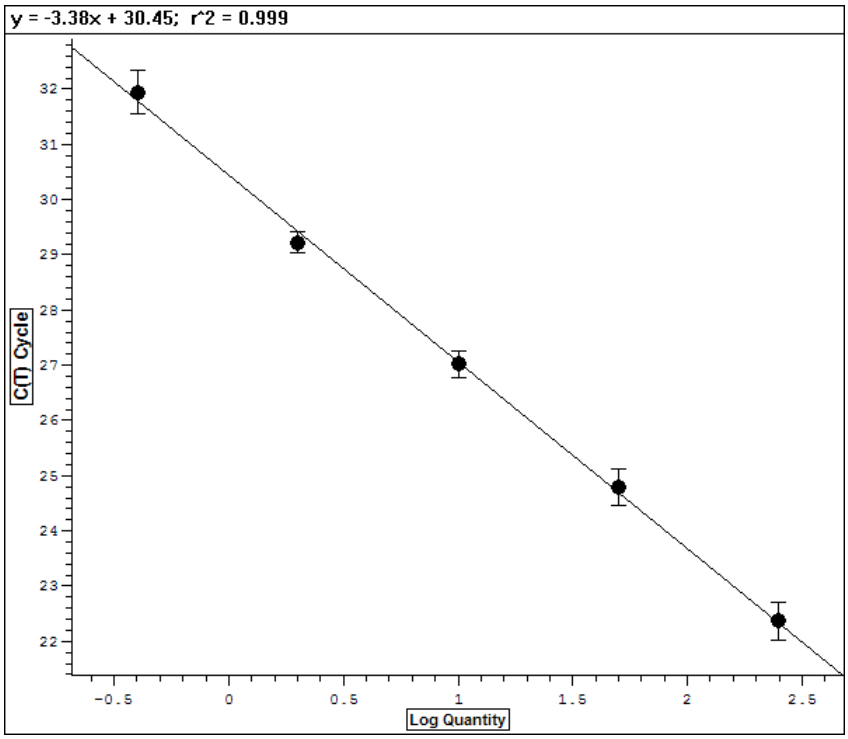

Figure 5b. Standard curve for dcytb. The graph depicts log of the quantity of cDNA on the x-axis and cycle threshold value on the y-axis for each of the standard samples used.

Figure 5c

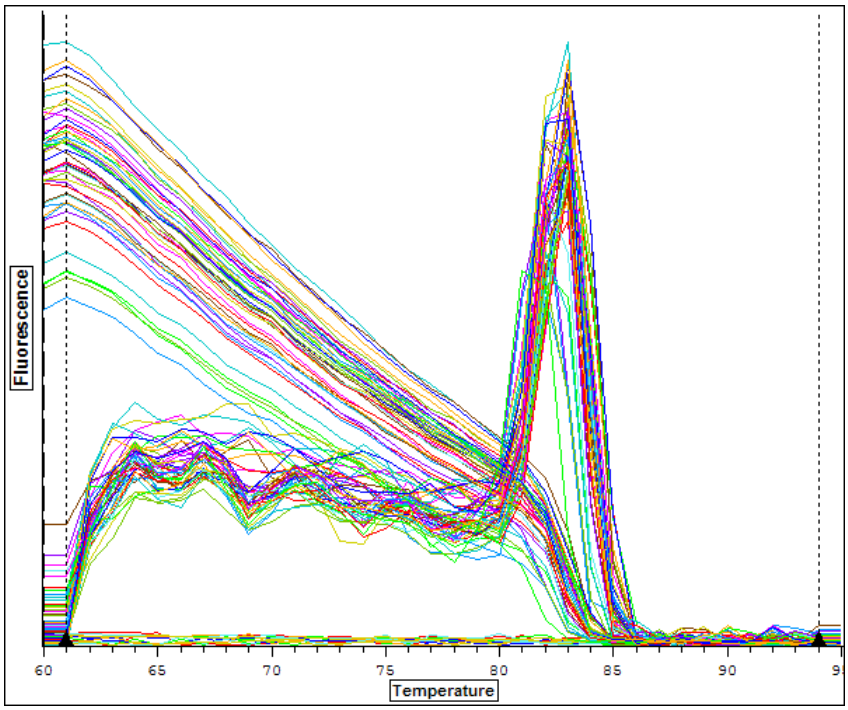

Figure 5c. Melting curve for dcytb. The graphical plot of the melting curve was obtained after the PCR, using dcytb-specific primers. The presence of a single peak at 82-83^{o}C is suggestive of the generation of a specific product during the PCR.

d) Hephaestin:

Figures 6a, b and c show the log fluorescence data, standard curve and melting curves respectively for hephaestin. The average efficiency of the PCR for hephaestin was found to be 96%. The melting curve analysis showed the presence of a single peak at 79-80^{o}C.

Figure 6a

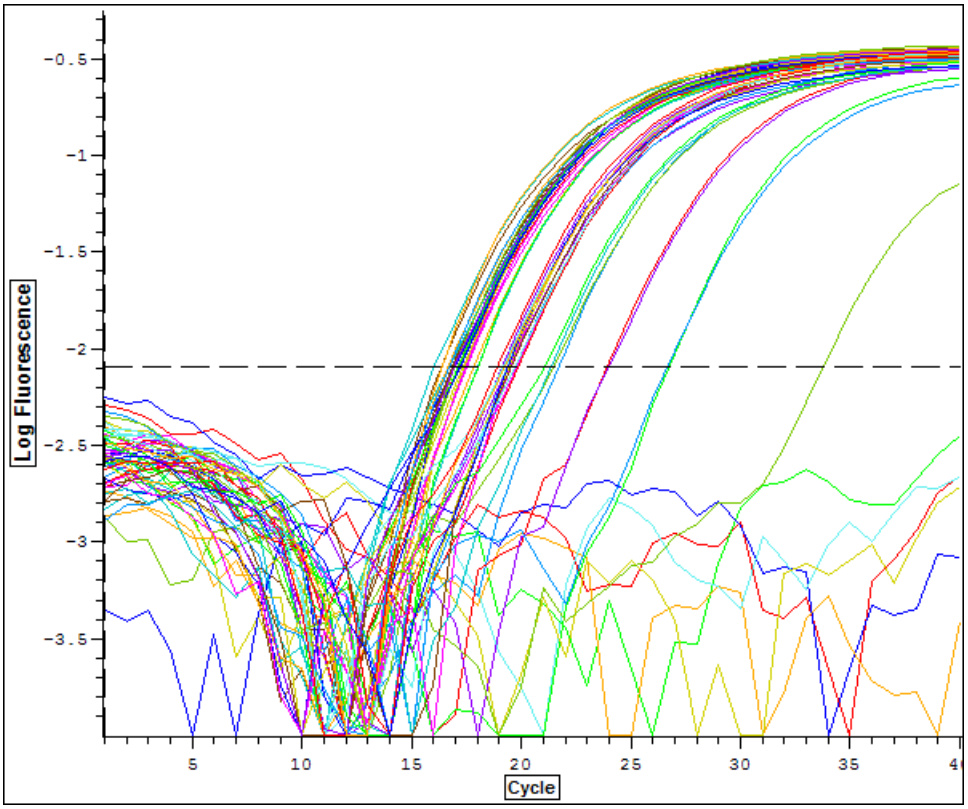

Figure 6a. Log fluorescence data graph for hephaestin. The graph depicts the PCR cycle number on the x-axis and relative log fluorescence units on the Y axis. The dotted line shows the cycle threshold is set at a point above baseline fluorescence.

Figure 6b

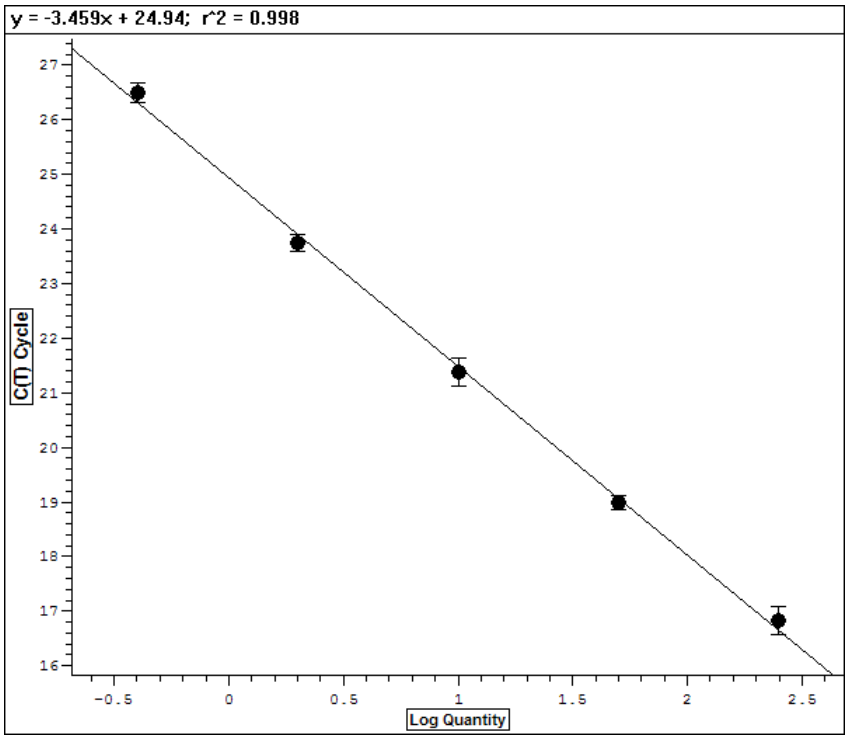

Figure 6b. Standard curve for hephaestin. The graph depicts log of the quantity of cDNA on the x-axis and cycle threshold value on the y-axis for each of the standard samples used.

Figure 6c

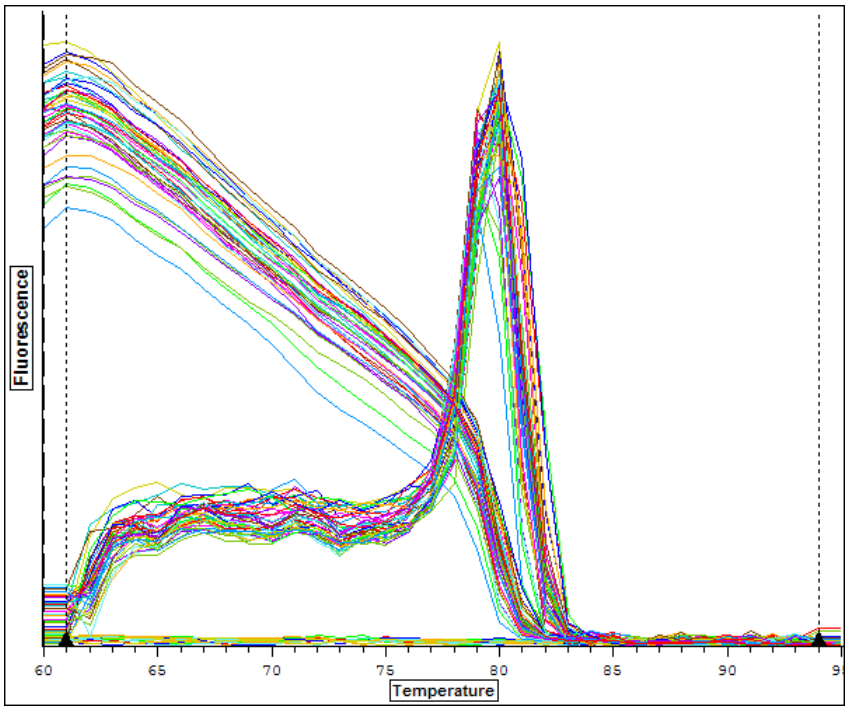

Figure 6c. Melting curve for hephaestin. The graphical plot of the melting curve was obtained after the PCR, using hephestin-specific primers. The presence of a single peak at 82-83^{o}C is suggestive of the generation of a specific product during the PCR.

e) Beta-actin:

Figures 7a, b and c show the log fluorescence data, standard curve and melting curves respectively for beta-actin. The average efficiency of the PCR for beta-actin was found to be 94%. The melting curve analysis showed the presence of a single peak at 79-80^{o}C.

Figure 7a

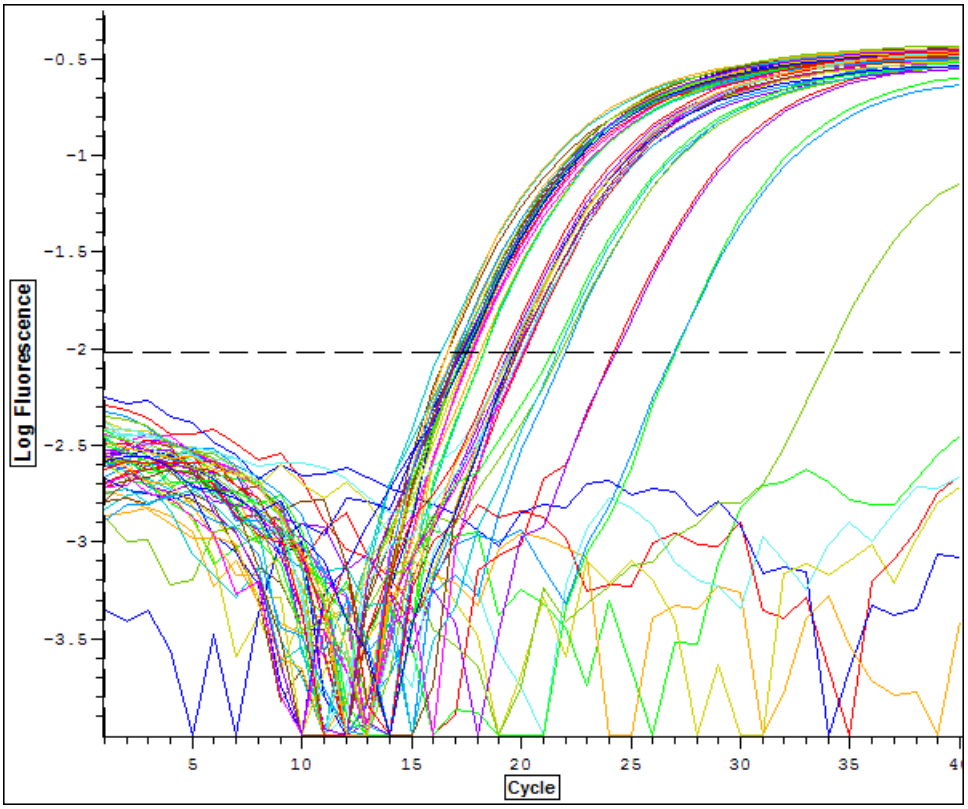

Figure 7a. Log fluorescence data graph for beta-actin. The graph depicts the PCR cycle number on the x-axis and relative log fluorescence units on the Y axis. The dotted line shows the cycle threshold is set at a point above baseline fluorescence.

Figure 7b

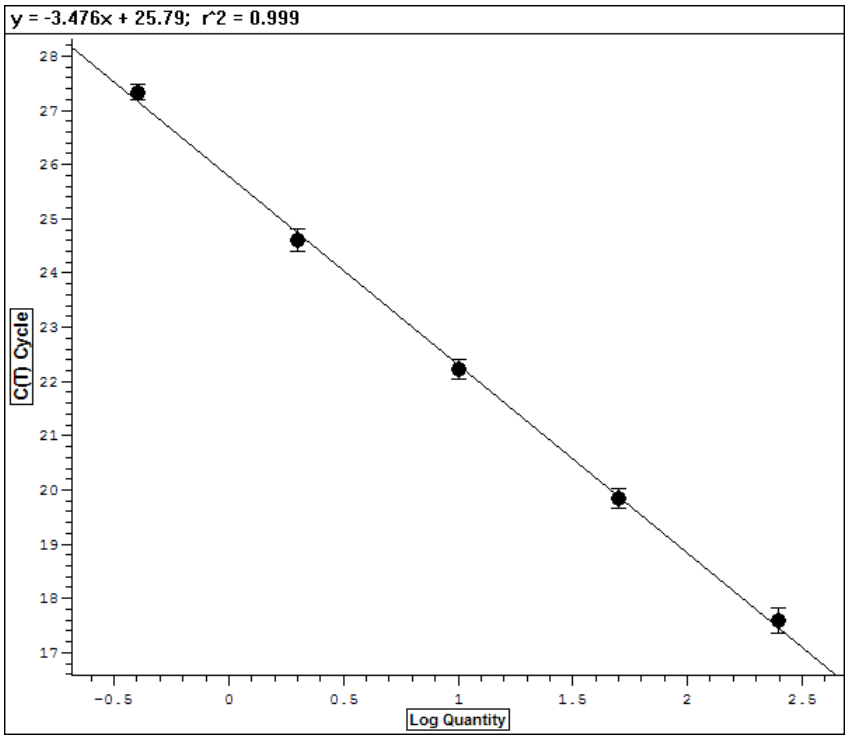

Figure 7b. Standard curve for beta-actin. The graph depicts log of the quantity of cDNA on the x-axis and cycle threshold value on the y-axis for each of the standard samples used.

Figure 7c

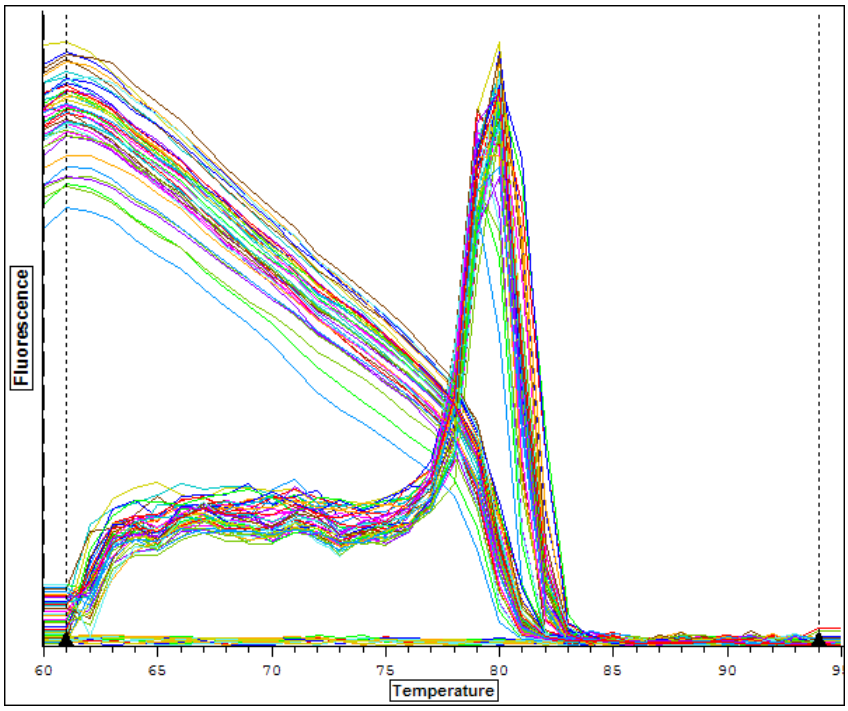

Figure 7c. Melting curve for beta-actin. The graphical plot of the melting curve was obtained after PCR, using dcytb-specific primers. The presence of a single peak at 80-81^{o}C is suggestive of the generation of a specific product during the PCR.

5. EXPRESSION OF DMT1 mRNA IN THE DUODENUM OF CONTROL AND ETHANOL-TREATED MICE

Fold-changes in levels of DMT-1 mRNA (relative to beta-actin) in the duodenum of control and ethanol-treated mice at 1, 2 and 4 weeks are shown in Figure 8. The expression of DMT-1 mRNA in the duodenum was significantly higher after 1 week of ethanol administration, as compared with the control mice in the same time period. There were no significant differences seen after 2 and 4 weeks of ethanol treatment.

Figure 8

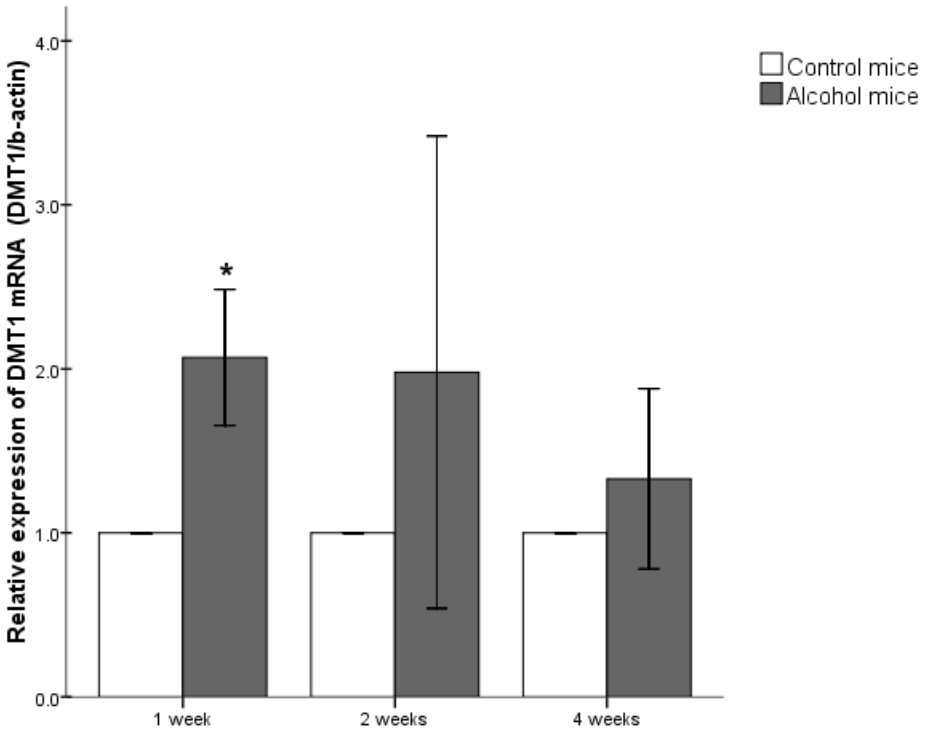

Figure 8. Relative expression of DMT1 mRNA in duodenum of control and alcohol-treated mice at the various time periods studied (N = 4 in each time period). Data was analyzed by the unpaired t-test to look for differences between control and alcohol-treated mice in each time period. ANOVA was used to look for differences among alcoholic mice at the different time periods studied. Data are shown as means $\pm$ SE. * indicates $p < 0.05$ when compared with corresponding control values.

6. EXPRESSION OF FERROPORTIN mRNA IN THE DUODENUM OF CONTROL AND ETHANOL-TREATED MICE

Fold change in ferroportin mRNA (relative to beta-actin) in the duodenum of control and the ethanol-treated mice are shown in Figure 9. The expression of ferroportin mRNA in the duodenum of the ethanol-treated mice was not significantly different from that in control mice after 1, 2 or 4 weeks of ethanol treatment.

Figure 9

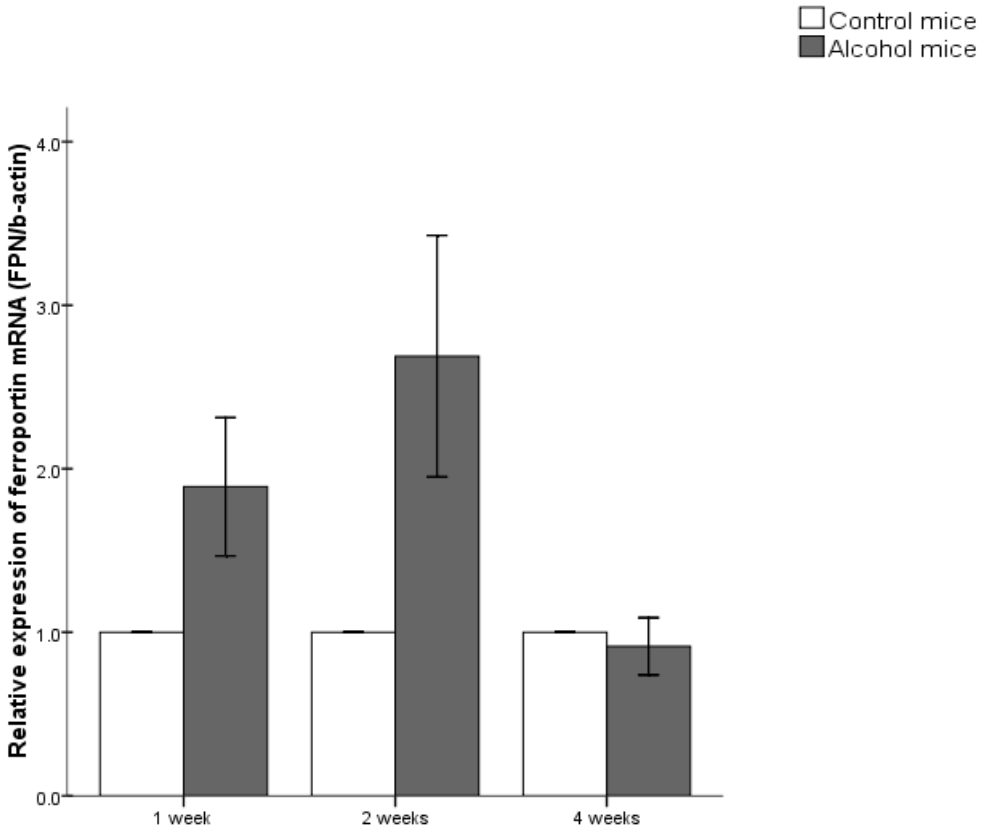

Figure 9. Relative expression of ferroportin mRNA in duodenum of control and alcohol-treated mice at the various time periods studied (N = 4 in each time period). Data was analyzed by the unpaired t-test to look for differences between control and alcohol-treated mice in each time period. ANOVA was used to look for differences among alcoholic mice at the different time periods studied. Data are shown as means $\pm$ SE.

7. EXPRESSION OF DUODENAL CYTOCHROME B (DCYTB) mRNA IN THE DUODENUM OF CONTROL AND ETHANOL-TREATED MICE

Fold changes in dcytb mRNA (relative to beta-actin) in the duodenum of control and ethanol-treated mice are shown in Figure 10. The expression of dcytb mRNA in the duodenum of the ethanol-treated mice was not significantly different from that in control mice after 1, 2 or 4 weeks of ethanol treatment.

Figure 10

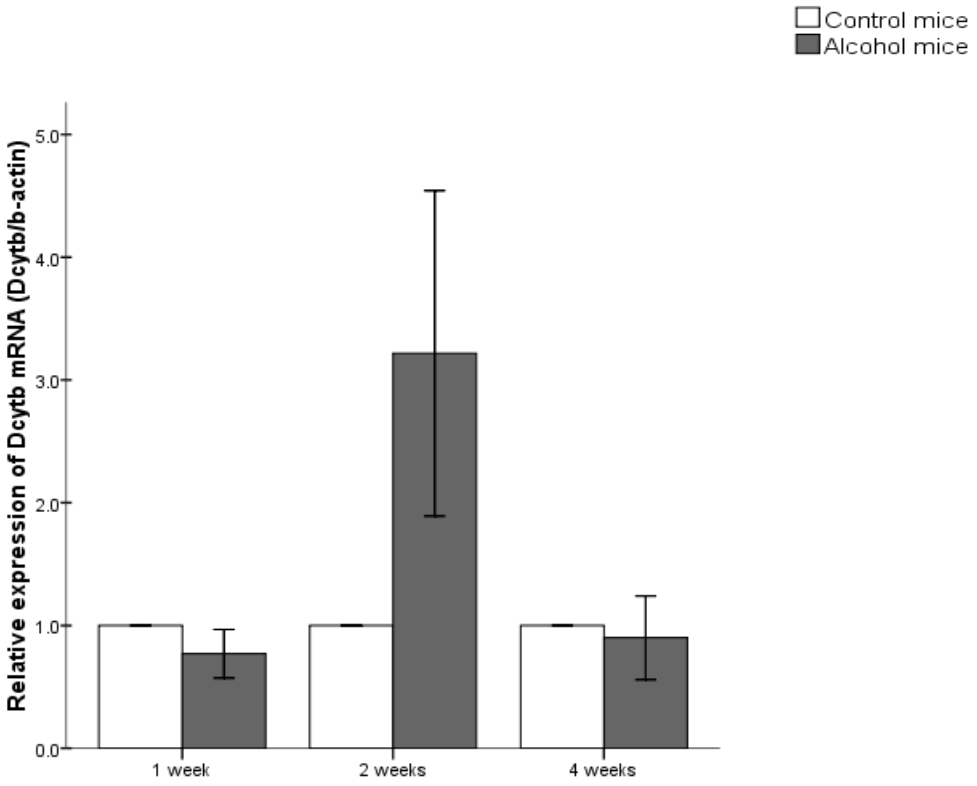

Figure 10. Relative expression of dcytb mRNA in duodenum of control and alcohol-treated mice at the various time periods studied (N = 4 in each time period). Data was analyzed by the unpaired t-test to look for differences between control and alcohol-treated mice in each time period. ANOVA was used to look for differences among alcoholic mice at the different time periods studied. Data are shown as means ± SE.

8. EXPRESSION OF HEPHAESTIN mRNA IN THE DUODENUM OF CONTROL AND ETHANOL-TREATED MICE

Fold changes in hephaestin mRNA (relative to beta-actin) in the duodenum of control and ethanol-treated mice are shown in Figure 11. The expression of hephaestin mRNA in the duodenum of the ethanol-treated mice was not significantly different from that in control mice after 1, 2 or 4 weeks of ethanol treatment.

Figure 11

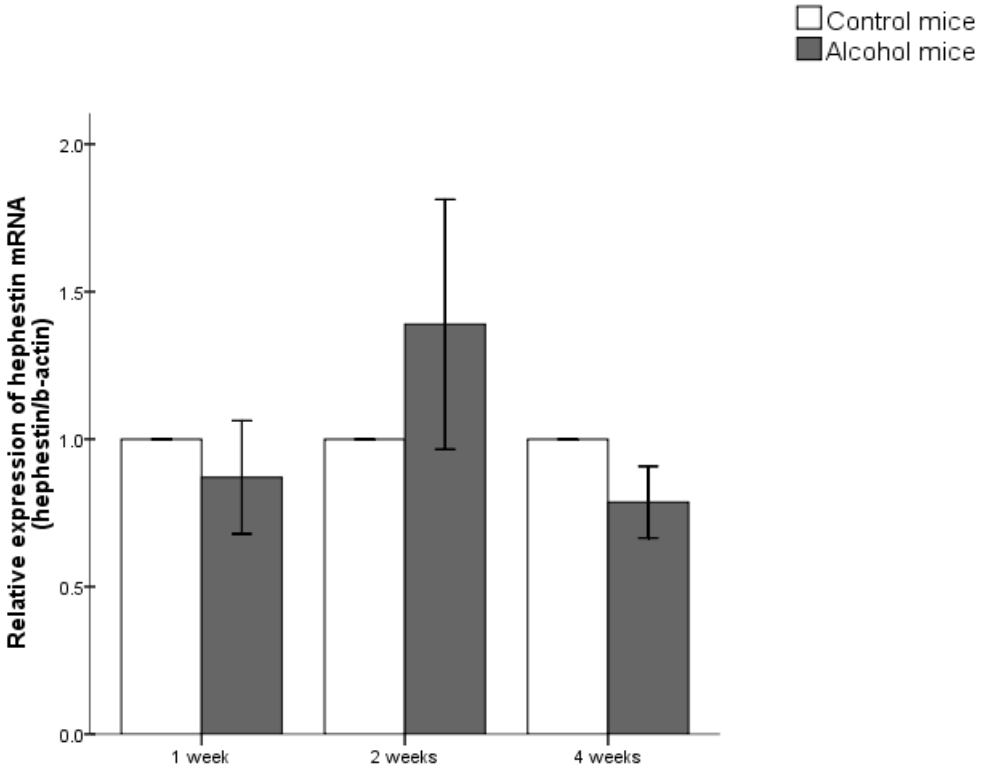

Figure11.Relative expression of hephaestin mRNA in duodenum of control and alcohol-treated mice at the various time periods studied (N=4 in each time period). Data was analyzed by the unpaired t-test to look for differences between control and alcohol-treated mice in each time period. ANOVA was used to look for differences among alcoholic mice at the different time periods studied. Data are shown as means $\pm$ SE.

9. EXPRESSION LEVELS OF DMT1 PROTEIN IN THE DUODENUM OF CONTROL AND ETHANOL-TREATED MICE

Figure 12 shows representative western blots for DMT1. Protein levels in the duodenum of the ethanol-treated mice were significantly higher than in corresponding control mice after 1 and 2 weeks of ethanol treatment (Figure 13). Levels of the protein were elevated after 4 weeks of ethanol treatment but the increase was not statistically significant.

Figure 12

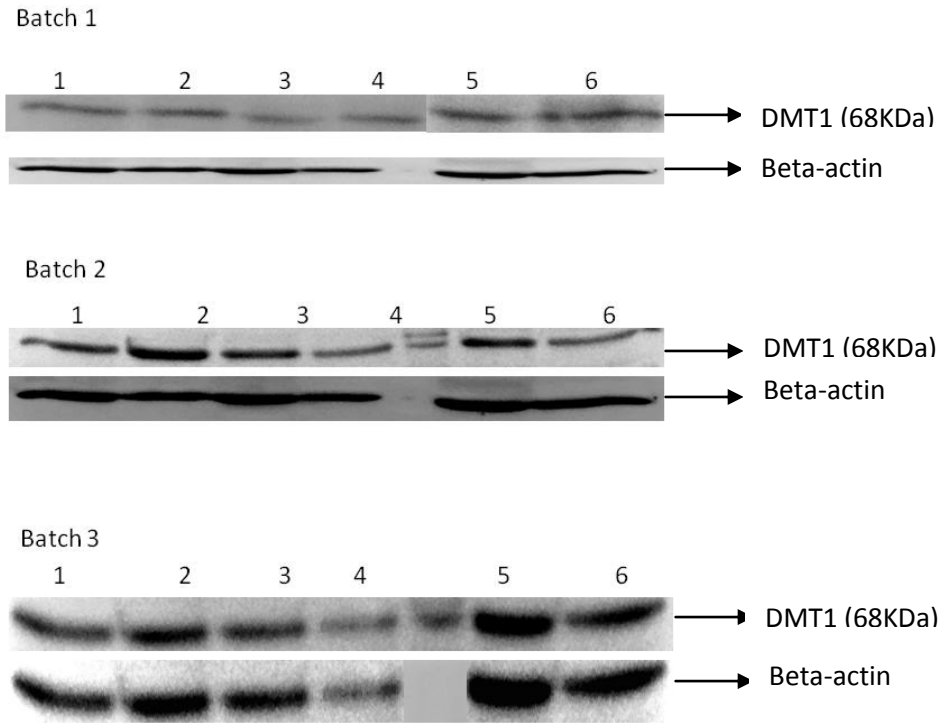

Figure 12: Western blots showing DMT1 (68 kDa) protein levels in control and alcohol-treated mice (N=3) over the different time periods studied. Lanes 1= 1 week control, 2 = 1 week alcohol treatment, 3 = 2 weeks control, 4 = 2 weeks alcohol treatment, 5 = 4 weeks controls and 6 = 4 weeks alcohol treatment Beta-actin was used as the loading control.

Figure 13

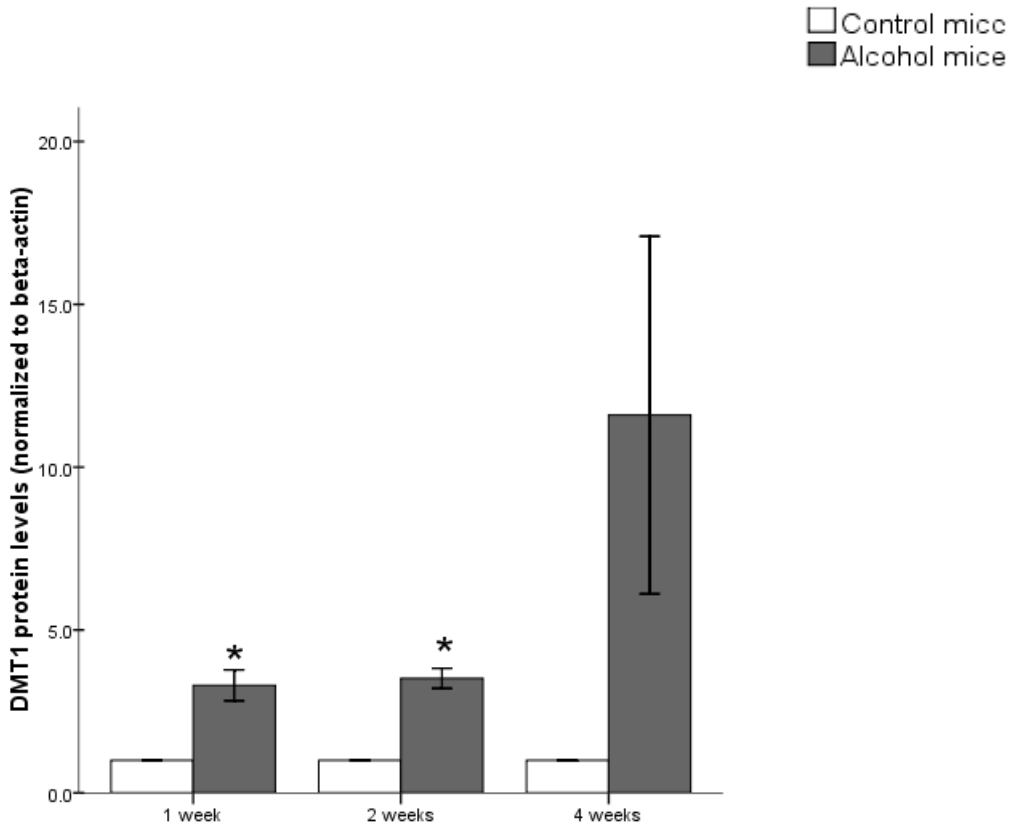

Figure 13. Densitometric quantification of bands of western blots for DMT1 in control and alcohol-treated mice at different time periods (N=3 in each time period). Data was analyzed by the unpaired t-test to look for differences between control and alcohol-treated mice in each time period. ANOVA was used to look for differences among alcoholic mice at the different time periods studied. Data are shown as means $\pm$ SE. * indicates $p < 0.05$ when compared with corresponding control values.

10. EXPRESSION LEVELS OF FERROPORTIN PROTEIN IN THE DUODENUM OF CONTROL AND ETHANOL-TREATED MICE

Figure 14 shows representative western blots for ferroportin. Protein levels in the duodenum of ethanol-treated mice were not statistically different from control mice at each of the time periods studied (Figure 15).

Figure 14

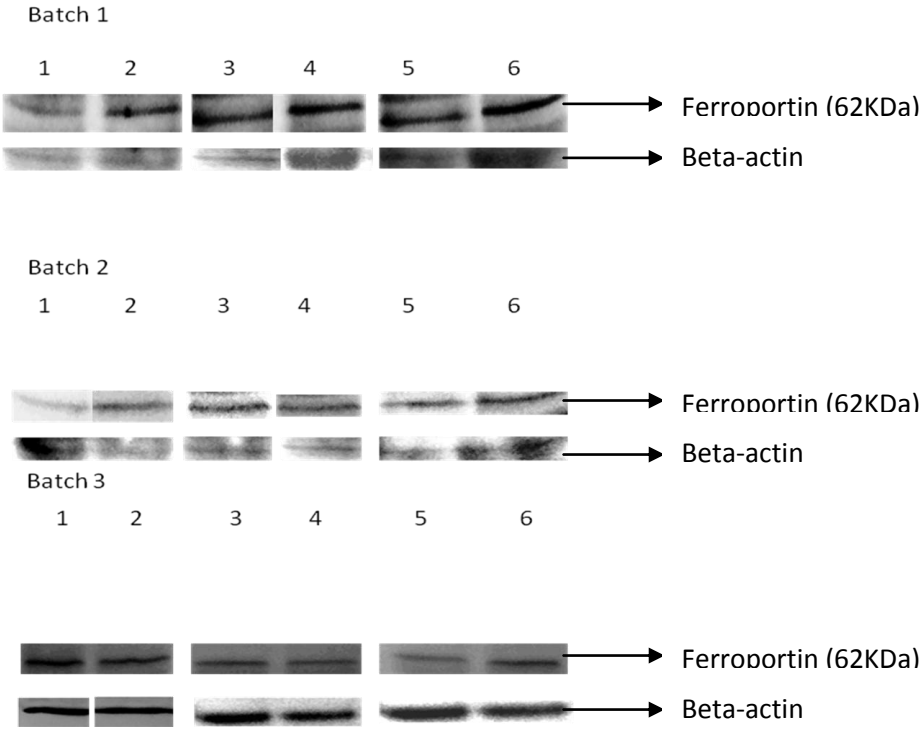

Figure 14: Western blots showing ferroportin (62 kDa) protein levels in control and alcohol-treated mice (N=3) over the different time periods studied. Lanes 1= 1 week control, 2 = 1 week alcohol treatment, 3 = 2 weeks control, 4 = 2 weeks alcohol treatment, 5 = 4 weeks controls and 6 = 4 weeks alcohol treatment Beta-actin was used as the loading control.

Figure 15

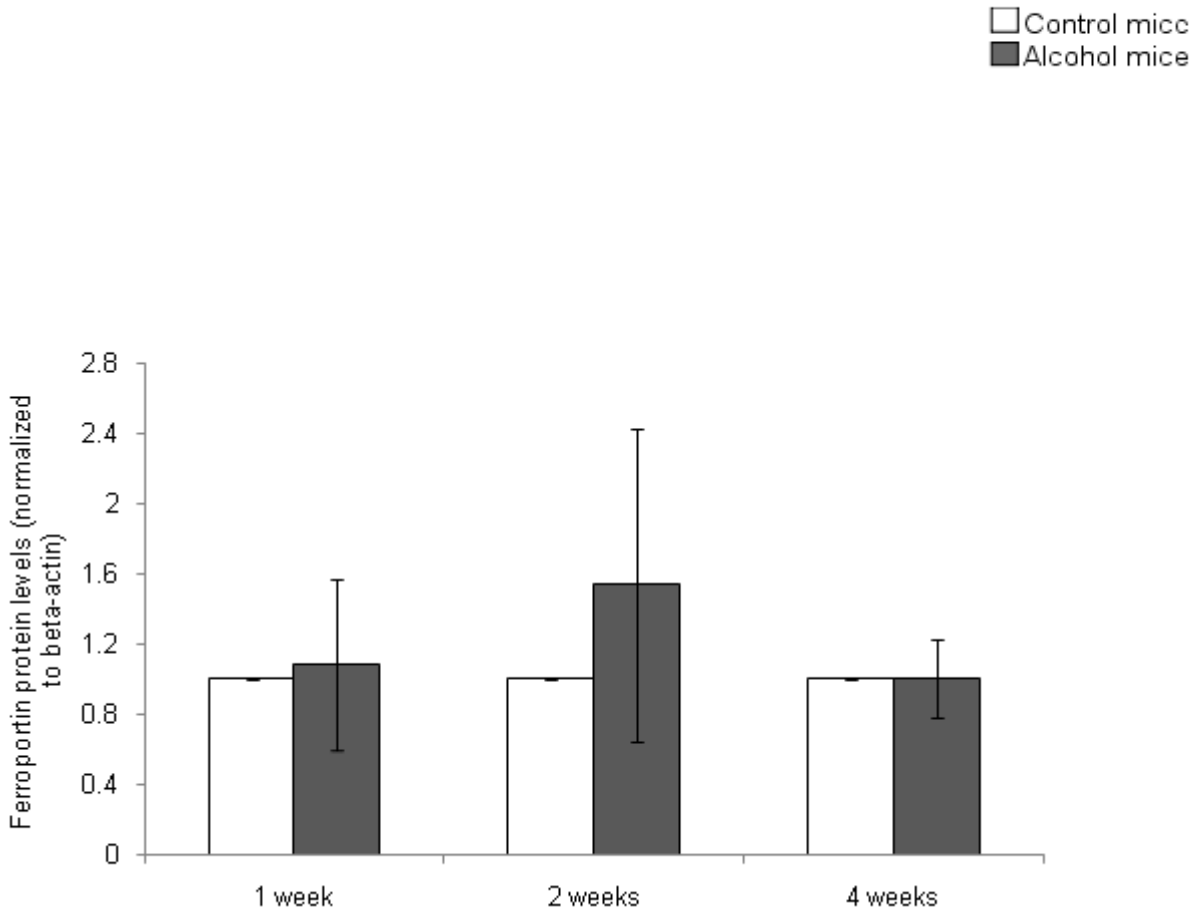

Figure 15. Densitometric quantification of bands of western blots for ferroportin in control and alcohol-treated mice at different time periods (N = 3 in each time period). Data was analyzed by the unpaired t-test to look for differences between control and alcohol-treated mice in each time period. ANOVA was used to look for differences among alcoholic mice at the different time periods studied. Data are shown as means $\pm$ SE.

11. ESTIMATION OF SERUM IRON IN CONTROL AND ETHANOL-TREATED MICE

Serum iron levels in control and alcohol-treated mice were not significantly different after 1, 2 and 4 weeks of ethanol treatment (Figure 16).

Figure 16

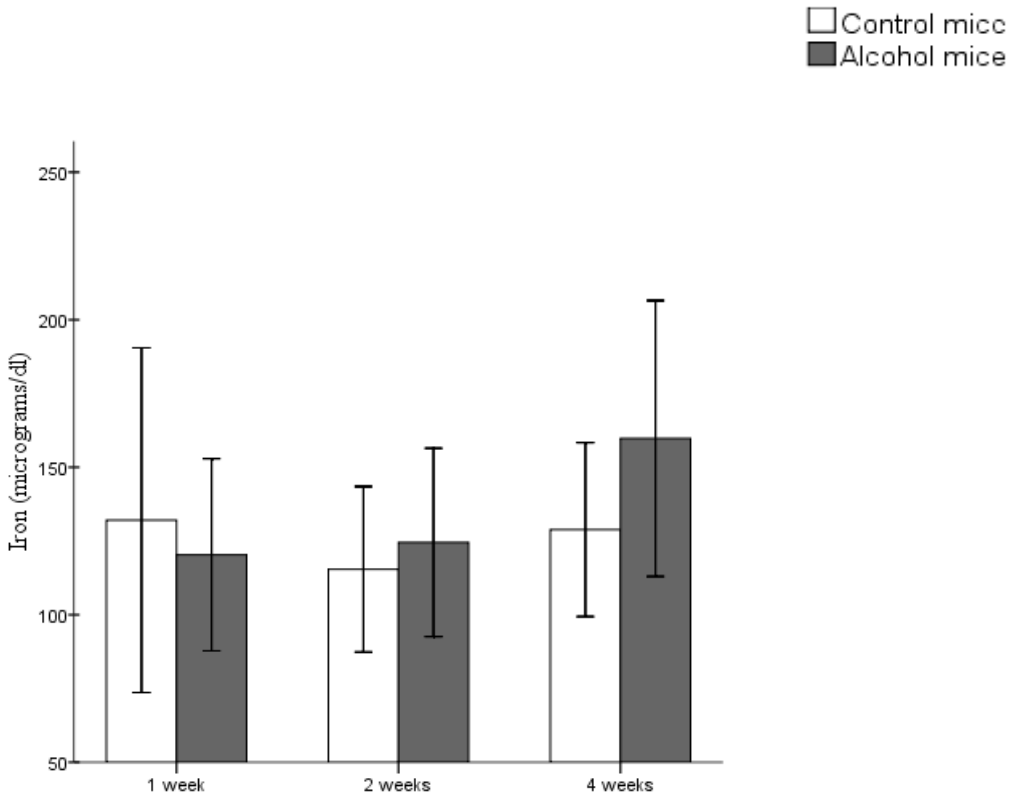

Figure 16. Levels of serum iron in control and alcohol-treated mice at different time periods studied (N = 4 in each time period). Data was analyzed by the unpaired t-test to look for differences between control and alcohol-treated mice in each time period. ANOVA was used to look for differences among alcoholic mice at the different time periods studied. Data are shown as means $\pm$ SE.

DISCUSSION

The field of iron metabolism has been transformed from an obscure area to one that is now built on robust ideas and insights. This field has seen a rapid transformation over the past two decades. Most of the studies on the mechanisms of iron handling by the body have come from patients with iron overload and deficiency, elegant studies on cells in culture and animal models of iron metabolism (Knutson, 2010) .

The current study was designed to ascertain the effect of alcohol ingestion on proteins involved in duodenal iron absorption. Male Swiss albino mice were chosen as the model for this study. Alcohol was administered for different time periods, using a commercially available alcohol-based diet, the Lieber-DeCarli diet. There are various ways to orally administer alcohol to experimental animals. One of them is to provide it in the drinking water. This method has some limitations. Animals are naturally averse to alcohol and this may lead to insufficient intake, resulting in inadequately sustained blood alcohol levels. This may make it difficult to correlate findings obtained with the situation encountered in clinical practice. When alcohol is supplemented in a liquid diet formula, such as in the Lieber-DeCarli diet, the intake of alcohol has been shown to be higher and leads to blood alcohol levels of clinical relevance in animal models of chronic alcoholism (Lieber et al., 1989). The Lieber-DeCarli diet is commercially available in alcohol and control diet forms. Another advantage of its use is that it allows alcohol to be added to the diet to replace varying proportions of the total calories given. This allows monitoring of calorie intake in both control and test animals. It facilitates comparison of alcohol-treated animals with control animals by simple pair-feeding. In addition, it is available as a powder, which can be easily blended in water. The disadvantages of its use are that it needs to be imported, requires storage at minus 20^{o}C and is expensive.

Pair-feeding is a method that ensures that animals receiving alcohol and control diets receive equal amounts of calories on a day-to-day basis (Caster and Armstrong, 1956). This is of importance, since malnutrition that may develop in the animals receiving alcohol is an important confounding factor in such experiments. In the current study, every animal receiving alcohol had a corresponding control animal that received the control Lieber-DeCarli diet that contained maltose dextrin instead of

alcohol. Each day, the control animal was provided with only as many calories as the alcohol- fed animal consumed in the previous 24 hours. Thus, it was ensured that both control and alcohol-fed mice received equal amounts of calories.

A change in the body weight is an important indicator of malnutrition in experimental mice (Ullman-Cullere and Foltz, 1999). Changes in the weights of alcohol-treated mice were not significantly different from those in pair-fed control mice over the time periods studied. Hence, it was concluded that both groups of mice had similar states of nutrition.

In this study, the volume of ethanol consumed per day by each mouse did not vary significantly over the study time periods of 1, 2 and 4 weeks. This indicates that the intake of alcohol by the mice had been, more or less, uniform throughout the periods of study. Thus, the duration of alcohol administration was the main variable factor among the groups.

Patients with alcoholic liver disease (ALD) often show evidence of iron overload, as assessed by determination of total iron content of liver or by estimation of serum transferrin saturation, which may be increased (Chapman et al., 1982, Ioannou et al., 2004). Serum ferritin concentrations may show a modest increase and there may be elevation of liver enzymes, such as gamma glutamyl transferase, alanine transaminase and aspartate transaminase (Whitfield et al., 2001). Consumption of even moderate amounts of alcohol has been associated with a modest increase in iron stores, particularly in liver, as demonstrated by histological studies on liver biopsies from alcoholic patients (Ioannou et al., 2004). Increased Tfr1 expression has also been demonstrated in hepatocytes of patients with ALD who have iron overload (Suzuki et al., 2002)

Iron is considered a secondary risk factor in the etiopathogenesis of hepatic injury in alcoholic liver disease (Irving et al., 1988). This is due to its ability to take part in redox reactions, such as the Fenton reaction, which can generate free radicals that damage tissues (Andrews and Schmidt, 2007). This action of iron is independent of, and in addition to, the damage inflicted by alcohol itself on the liver.

Iron is absorbed from intestine (~1-2mg/day) and obtained from recycling of body stores (mainly from senescent red blood cells) by macrophages (~25mg/day) (Knutson, 2010). There is some evidence that intestinal iron transport is increased in animal models of alcohol ingestion (Kohgo et al., 2008). Increased iron load in the body has been shown to trigger synthesis of hepcidin which binds to ferroportin (the cellular exporter of iron), which is then internalized and degraded intracellularly (Nemeth et al., 2004b). These effects, seen in macrophages and cancer cell lines, have been extrapolated to enterocytes (Mena et al., 2008). However, enterocytes appear to be less sensitive to regulation by hepcidin, as compared with effects in macrophages, since levels of ferroportin were not found to be affected by exposure to hepcidin (Mena et al., 2008, Chaston et al., 2008).

The current study shows that expression of DMT1 was significantly higher at the end of weeks 1 and 2 after alcohol treatment, while expression of ferroportin was unaffected. Harrison-Findik et al (2006) have reported that protein levels of both DMT1 and ferroportin were increased in alcohol-treated mice after 1 week of treatment. Several reasons may account for the differences between the current study and those of Harrison-Findik et al (2006). They used 6 weeks-old male and female 129/Sv mice, weighing 15-20g, in their study, while the current study used male Swiss albino mice (~30g), at 3-4 months of age. 129/Sv mice are inbred and thus genetically more homogeneous than the Swiss-albino mice. This may account for greater biological variations in mice in the current study. In addition, Harrison-Findik et al (2006) have used both female and male mice. They have shown that there is a significant gender difference in hepcidin production in male and female 129/SV mice, with female mice producing more hepcidin than the males. Since their data showed combined findings for males and females, it is possible that the female mice, with higher expression levels of hepcidin, showed a greater suppression of ferroportin as compared to the male mice. The mean amounts of alcohol consumed by mice in their study were in the range of 0.36 to 0.52 ml per mouse per day, which was comparable to the amounts consumed by mice in the current study. However, the mice in the study of Harrison-Findik et al (2006) were given alcohol in drinking water, at concentrations of 10% or 20% for 7 days. This route of administration is not the best way to study

the effect of alcohol on an animal, as it is difficult to accurately quantitate the amount of alcohol the mice have consumed under these conditions. The use of the Lieber-DeCarli diet is a better way to administer alcohol to rodents. In addition, the current study has made use of a commercially available rabbit anti-ferroportin antibody while the mouse anti-ferroportin antibody used by Harrison-Findik et al (2006) was generated in their own laboratory. Most importantly, the molecular weight of the protein they have detected with their antibody is 78kDa, while the molecular weight of the protein detected with the commercially available antibody was 62 kDa, which is the molecular weight that is most commonly reported for ferroportin (Rice et al, 2009; Mena et al 2007; Swiss protein repository (accessible at uniprot.org/uniprot/Q9JHI9)). In the work of Rice et al (2009), characterizing the structure and function of ferroportin in mammalian cells, they have reported a single predominant band migrating with an apparent molecular mass of ~ 60 kDa. The Swiss protein repository (accessible at uniprot.org/uniprot/Q9JHI9) states that the molecular weight of mouse ferroportin is 62.7 kDa. In addition, the NCBI protein database clearly lists mouse ferroportin as a protein with a molecular weight 62.5KDa (accession no. GenBank: AAK77858.2). In view of these reports, it is reasonably certain that the protein band detected in the current study is indeed ferroportin. All of these reasons could contribute to the differences seen in the effect of alcohol on ferroportin in the two studies.

There are very few animal or human studies on the effect of alcohol on DMT1 and ferroportin in enterocytes. Studies have shown that that co-administration of dietary alcohol and iron in rats led to liver injury and hepatic iron overload (Tsukamoto et al., 1995; Valerio et al., 1996). However, the mechanism by which this occurred was not reported. Another study in humans has proposed the possibility of increased intestinal iron absorption in patients with ALD but this aspect was not investigated further (Kohgo et al., 2005). Thus, further work is warranted to better elucidate the mechanisms by which iron overload in ALD.

Harrison-Findik et al (2006) have shown that alcohol can regulate hepcidin gene transcription. In their study, ethanol was found to down-regulate both the hepcidin promoter activity and the DNA-binding activity of C/EBP alpha transcription factor. This would lead to the down-regulation of liver

hepcidin gene transcription, and thereby increased duodenal iron transport. Other studies have reported that Caco-2 cells treated with hepcidin were found to express lower levels of DMT1 mRNA and protein levels with no changes in ferroportin mRNA and protein levels (Mena et a, 2008; Yamaji et al., 2004). This was associated with decreased apical uptake of iron with no change in basolateral transfer of iron, findings that were also seen in studies with rat duodenal segments (Mena et al, 2008; Laftah et al, 2004). These reports are indicative of hepcidin affecting DMT1 directly, rather than through its effect on ferroportin. It would necessary to determine the effect of alcohol on hepatic hepcidin and in the mouse model in the current study to see if such effects are operational in this study. It has not been possible to determine the expression of hepatic hepcidin in the current study so far. However, the study is on-going and studies on hepcidin expression will be carried out shortly. It would also be desirable to estimate serum levels of hepcidin in the experimental mice. However, as far as known, there are no reagents available commercially for this estimation. This poses a major limitation.

Most of dietary iron is in the ferric form and has to be reduced to the ferrous form before being transported into the enterocytes by DMT1.The major reductant enzyme for this purpose has been reported to be dcytb (Andrews and Schmidt, 2007). Studies have suggested that dcytb is strongly regulated by iron (McKie et al., 2001). Increased expression of this protein was found to increase iron absorption in enterocytes (Latunde-Dada et al., 2008). Dcytb has also been found to be a target of hypoxia-indicible factor 2α (HIF-2α). Genetic disruption of HIF signalling was found to disrupt increased iron absorption that occurred in response to iron deficiency in mice (Shah et al., 2009). Mutations of dcytb in cybrd null mice, however, failed to show any phenotypic alterations, with the affected mice not showing changes in iron metabolism (Gunshin et al., 2005). Ohgami et al (2006) have suggested the presence of other ferrireductases of the STEAP (Six Transmembrane Epithelial Antigen of the Prostate) family in enterocytes. These results suggest that dcytb may not be indispensible for reduction of dietary iron. The current study shows that dcytb levels were unaffected by alcohol administration for up to 4 weeks. Increased expression levels of DMT1 without corresponding changes in dcytb may be due to the presence of other enzymes with ferrireductase

activity in the enterocytes, as has been reported. As far as has been ascertainable, there are no studies that have reported the effect of alcohol on dcytb.

In the current study, hephaestin expression in the mouse duodenum was found to be unaffected by alcohol administration for up to 4 weeks. There are no reports to date that have studied this effect. Hephaestin is a copper dependent enzyme in the basal border of enterocytes, which helps in oxidizing ferrous iron to ferric iron so that it can be loaded onto transferrin in circulation for transport to iron-requiring cells (Vulpe et al., 1999). Hephaestin-deficient mice have been shown to have anemia and mucosal retention of iron (Vulpe et al., 1999). Most of these studies on hephaestin had been performed on sex-linked anaemia (*sla*) mice. It has been now been shown that hephaestin is not absolutely essential for iron absorption, as these *sla* mice, though anaemic at birth, manage to grow and reproduce with their anaemia resolving with time (Vulpe et al., 1999). Ceruloplasmin-null mice have defective iron absorption when the iron homeostatic pathway was stressed by phlebotomy (Cherukuri et al., 2005). It is thus possible that ceruloplasmin can partially overcome the effects of hephaestin defiency (Knutson, 2010). Studies on patients of genetic hemochromatosis have revealed that hephaestin mRNA and protein levels are not altered in states of iron overload (Gleeson et al., 2005, Rolfs et al., 2002, Stuart et al., 2003, Zoller et al., 2003). These reports suggest that hephaestin is not indispensible for iron homeostasis and are likely to explain the findings in the current study.

Levels of plasma iron have a role to play in maintenance of iron homeostasis in the body. Homeostatic mechanisms involved are integrated to maintain an adequate saturation of transferrin with iron. Such mechanisms respond to pathways that consume iron and pathways that supply iron (Knutson, 2010). The current study shows that serum iron tended to increase after 2 and 4 weeks of alcohol treatment. The increases were, however, not statistically significant. In patients with ALD, evidence of elevated iron stores is indicated by iron indices such as levels of serum iron, ferritin and transferrin saturation. Levels of serum iron in patients with ALD were not found to be different from alcoholics who had no evidence of liver disease (Machado et al., 2009). Valimaki et al (Valimaki et al., 1983) showed that serum iron levels increased immediately after a bout of drinking in chronic

alcoholics but returned to normal after a week. They found no elevation of serum iron levels after acute consumption of alcohol by normal healthy controls. On the other hand, Whitfield et al (2001) showed that serum iron levels progressively increased with increasing alcohol intake. Thus, studies differ in their findings with regard to the effect of alcohol ingestion on serum iron levels.

SUMMARY

The preliminary results of this study showed that administration of ethanol caused up-regulation of DMT1 (the apical iron transporter), in the mouse duodenum after 1 and 2 weeks of treatment. Ferroportin (the basolateral iron exporter), dcytb (the apical ferrireductase) and hephaestin (the basolateral ferroxidase) were found to be unaffected by the treatment. Alcohol administration for the periods of study did not cause significant alterations in serum iron levels at the time periods studied.

LIMITATIONS OF THE STUDY

1. It has not been possible, within the time frame of the current study, to determine the effect of alcohol on hepatic hepcidin expression. This will, however, be carried out shortly.
2. It has not been possible to measure serum hepcidin levels, due to non-availability of commercially available reagents to measure it in mouse serum.
3. It has, thus, not been possible to correlate the findings of this study with levels of hepcidin, which is known to be a central regulator of iron homeostasis. .

FUTURE DIRECTIONS

There are several parameters that will need to be studied in order to elucidate more clearly the mechanisms involved in iron load in response to ingestion of alcohol. These are:

1. Hepatic and serum hepcidin levels
2. Expression levels of ferroportin and DMT1 in the liver
3. Ferritin levels in blood, duodenum and liver
4. Iron levels in the liver
5. Histopathological studies to look for changes in the liver in response to alcohol administration and for evidence of iron overload.
6. Expression levels of protein for duodenal dcytb and hephaestin
7. Expression levels of transferrin receptors 1 and 2 in the liver

Data obtained from these further studies are likely to contribute to knowledge to answer questions in the area of iron overload in alcoholic liver disease.

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APPENDIX

Table A1: Data for weight change in alcohols and control mice in 1 week:

Animal Number	**1C6**	**1A6**	**1C7**	**1A7**	**1C8**	**1A8**	**1C9**	**1A9**
0 days	32	31	30	25	29	31	25	26
1 day	30	28	29	26	29	33	25	27
7 days	30	28	30	25	27	31	26	27

Table A2: Data for weight change in alcohols and control mice in 2 weeks:

Animal Number	**2C9**	**2A9**	**2C10**	**2A10**	**2C11**	**2A11**	**2C8**	**2A8**
0 day	29	29	30	29	29	28	37	28
1 week	29	26	29	26	30	27	37	29
2 weeks	29	28	32	28	30	29	39	30

Table A3: Data for weight change in alcohols and control mice in 4 weeks:

Animal Number	**4C6**	**4A6**	**4C8**	**4A8**	**4C9**	**4A9**	**4C10**	**4A10**
0 day	26	30	31	28	30	31	28	30
1 week	24	24	28	25	31	33	29	29
2 week	22	23	28	20	31	34	30	30
3 weeks	22	25	27	22	32	35	28	31
4 weeks	24	27	29	25	32	34	29	29

Table A4: Data for volume of Lieber-DeCarli diet and alcohol consumed (in ml) in 1 week

1 week										
	Batch 5	alcohol(ml)	Batch 6	alcohol(ml)	Batch 7	alcohol(ml)	Batch 8 T	alcohol(ml)	Batch 9	alcohol(ml)
day 1	9	0.3364615	11	0.4112308	10	0.3796923	13	0.486	12	0.4486154
day 2	12	0.4486154	13	0.486	10	0.3796923	14	0.5276923	13	0.49
day 3	12	0.4486154	11	0.4112308	11	0.4176615	15	0.5653846	11	0.4146154
day 4	12	0.4486154	14	0.5233846	13	0.4936	16	0.6030769	15	0.5653846
day 5	13	0.486	14	0.5233846	8	0.3037538	15	0.5653846	11	0.4146154
day 6	13	0.486	14	0.5233846	15	0.5695385	13	0.49	11	0.4146154
day 7	9	0.3364615	11	0.4112308	14	0.5315692	11	0.4146154	10	0.3769231
mean	11.42857	**0.4272527**	12.57143	**0.469978**	11.57143	**0.4393582**	13.85714	**0.5217363**	11.85714	**0.4463956**
sd	1.718249	**0.0642361**	1.511858	**0.0565202**	2.507133	**0.0951939**	1.676163	**0.0635365**	1.676163	**0.0631414**

Table A5: Data for volume of Lieber-DeCarli diet and alcohol consumed (in ml) in 2 weeks

2 weeks								
	Batch 8		Batch 9		Batch 10		Batch 11	
day 1	11	0.4112308	13	0.486	12	0.4486154	10	0.3738462
day 2	8	0.2990769	13	0.486	15	0.5653846	10	0.3769231
day 3	9	0.3364615	10	0.3738462	13	0.49	11	0.4146154
day 4	13	0.486	12	0.4486154	15	0.5653846	10	0.3769231
day 5	12	0.4486154	13	0.486	14	0.5276923	10	0.3769231
day 6	12	0.4486154	16	0.5981538	12	0.4523077	16	0.6030769
day 7	12	0.4486154	12	0.4486154	14	0.5276923	11	0.4146154
day 8	15	0.5607692	12	0.4486154	13	0.49	11	0.4146154
day 9	15	0.5607692	15	0.5607692	12	0.4523077	11	0.4146154
day 10	11	0.4112308	10	0.3738462	11	0.4146154	16	0.6030769
day 11	15	0.5607692	15	0.5607692	14	0.5276923	12	0.4523077
day 12	10	0.3738462	20	0.7476923	12	0.4523077	12	0.4523077
day 13	10	0.3738462	12	0.4486154	14	0.5276923	12	0.4523077
day 14	10	0.3738462	11	0.4112308	10	0.3769231	12	0.4523077
average	11.64286	**0.4352637**	13.14286	**0.4913407**	12.92857	**0.487044**	11.71429	**0.4413187**
sd	2.239751	**0.0837322**	2.656115	**0.0992978**	1.491735	**0.0564121**	1.9779	**0.074761**

Table A6: Data for volume of Lieber-DeCarli diet and alcohol consumed (in ml) in 4 weeks

	4 weeks							
Days	Batch6	alcohol	Batch 8	alcohol	Batch 9	alcohol	Batch 10	alcohol
1	7	0.261692	8	0.299077	18	0.672923	15	0.560769
2	10	0.373846	9	0.336462	18	0.678462	14	0.527692
3	7	0.261692	6	0.224308	15	0.565385	12	0.452308
4	8	0.299077	6	0.224308	19	0.716154	13	0.49
5	7	0.261692	8	0.299077	18	0.678462	12	0.452308
6	5	0.186923	6	0.224308	18	0.678462	14	0.527692
7	4	0.149538	4	0.149538	19	0.716154	14	0.527692
8	5	0.186923	5	0.186923	17	0.640769	15	0.565385
9	5	0.186923	4	0.149538	20	0.753846	14	0.527692
10	6	0.224308	7	0.261692	18	0.678462	17	0.640769
11	5	0.186923	6	0.224308	16	0.603077	12	0.452308
12	10	0.373846	5	0.186923	16	0.603077	13	0.49
13	5	0.186923	4	0.149538	20	0.753846	12	0.452308
14	7	0.261692	4	0.149538	20	0.753846	15	0.565385
15	3	0.112154	6	0.224308	13	0.49	14	0.527692
16	3	0.112154	7	0.261692	14	0.527692	15	0.565385
17	6	0.224308	7	0.261692	15	0.565385	15	0.565385
18	6	0.224308	5	0.186923	13	0.49	12	0.452308
19	5	0.186923	5	0.186923	15	0.565385	15	0.565385
20	5	0.186923	6	0.224308	13	0.49	14	0.527692
21	6	0.224308	5	0.186923	13	0.49	15	0.565385
22	7	0.261692	4	0.149538	12	0.452308	11	0.414615
23	15	0.560769	11	0.411231	12	0.452308	12	0.452308
24	10	0.373846	3	0.112154	10	0.376923	17	0.640769
25	13	0.486	14	0.523385	14	0.527692	12	0.452308
26	14	0.523385	18	0.672923	15	0.565385	14	0.527692
27	10	0.373846	13	0.486	16	0.603077	11	0.414615
28	15	0.560769	16	0.598154	12	0.452308	11	0.414615
		0		0		0		0

Mean	7.46	0.281346	7.21	0.271923	15.68	0.590962	13.57	0.511538
Sd	3.43	0.129145	3.82	0.144116	2.83	0.106636	1.69	0.063594

Table A7: Fold change of DMT1 RNA in alcohol mice over control mice by RT-PCR (1 week batch)

		ANIMAL NO.	1C9	1C6	1C7	1C8		
1 week	Control	DMT1	28.28	24.03	24.29	23.15		
		B actin	23.36	18.52	17.95	16.88		
		dct(ctGOI-ctREF)	4.92	5.51	6.34	6.27	**mean**	**sd**
		rel gene expression	0.03303 1814	0.02194 4451	0.01234 4395	0.01295 8118	**0.02**	**0.01**
		ANIMAL NO.	1A9	1A6	1A7	1A8		
1week	Alcohol	DMT1	27.24	22.77	22.5	22.59		
		b actin	23.13	17.58	17.84	17.37		
		dct(ctGOI-ctREF)	4.11	5.19	4.66	5.22	**Mean**	**sd**
		rel gene exprssion	0.05791 1754	0.02739 3929	0.03955 4894	0.02683 017	**0.04**	**0.01**
Fold change in alcohols over controls			1.75321 1443	1.24833 0549	3.20427 951	2.07052 9848	2.07	0.83

Table A8: Fold change of DMT1 RNA in alcohol mice over control mice by RT-PCR (2 weeks batch)

		ANIMAL NO.	2C9	2C10	2C11	2C8		
2 weeks	Control	DMT1	28.71	26.86	23.29	24.99		
		B actin	22.08	23.11	22.2	20.18		
		dct(ctGOI-ctREF)	6.63	3.75	1.09	4.81	**mean**	**sd**
		rel gene expression	0.01009 6506	0.07432 5445	0.46976 1375	0.03564 8866	**0.15**	**0.22**
		ANIMAL NO.	2A9	2A10	2A11	2A8		
2weeks	Alcohol	DMT1	26.53	27.05	27.7	23.77		
		b actin	22.55	22.52	24.01	18.8		
		dct(ctGOI-ctREF)	3.98	4.53	3.69	4.97	**Mean**	**sd**
		rel gene exprssion	0.06337 2467	0.04328 4671	0.07748 1731	0.03190 6629	**0.05**	**0.02**
Fold change in alcohols over controls			6.27667 2783	0.58236 6793	0.16493 8489	0.89502 5071	1.98	2.88

Table A9: Fold change of DMT1 RNA in alcohol mice over control mice by RT-PCR (4 weeks batch)

		ANIMAL NO.	4C9	4C6	4C10	4C8	Mean	sd
4 weeks	Control	DMT1	29.5	26.56	27.84	28.06		
		B actin	24.15	20.97	24.01	21.77		
		dct(ctGOI-ctREF)	5.35	5.59	3.83	6.29	**mean**	**sd**
		rel gene expression	0.024518253	0.020760716	0.070316155	0.01277972	**0.03**	**0.03**
		ANIMAL NO.	4A9	4A6	4A10	4A8		
4weeks	Alcohol	DMT1	26.05	26.93	28.01	26.82		
		b actin	22.2	21.82	22.32	20.24		
		dct(ctGOI-ctREF)	3.85	5.11	5.69	6.58	**Mean**	**sd**
		rel gene exprssion	0.069348092	0.028955877	0.019370433	0.010452559	**0.03**	**0.03**
Fold change in alcohols over controls			2.828427125	1.394743666	0.275476279	0.817902059	1.33	1.10

Table A10: Fold change of ferroportin mRNA in alcohol mice over control mice by RT-PCR (1 weeks batch)

Animal No.	1C9	1C6	1C7	1C8	**Mean**	**sd**
B ACTIN	23.36	18.52	17.95	16.88		
Fpn	27.3	22.02	22.15	20.24		
dct (ctGOI-ctBACT)	3.94	3.5	4.2	3.36		
rel gene expression	0.06515411	0.088388348	0.05440941	0.097395572	**0.07**	**0.02**
	1A9	1A6	1A7	1A8		
B ACTIN	23.13	17.58	17.84	17.37		
Fpn	25.61	20.32	20.83	21.05		
dct (ctGOI-ctBACT)	2.48	2.74	2.99	3.68		
rel gene expression	0.179244406	0.149684838	0.125869444	0.078020659	**0.15**	**0.03**
					Mean	**sd**
Fold change in alcohols over controls	2.751083636	1.693490625	2.313376368	0.801069878	**1.89**	**0.8**

Table A11: Fold change of ferroportin mRNA in alcohol mice over control mice by RT-PCR (2 weeks batch)

Animal No.	2C9	2C10	2C11	2C8	**Mean**	**sd**
B ACTIN	20.12	22.55	21.53	20.18		
Fpn	23.78	27.35	25.74	23.85		
dct (ctGOI-ctBACT)	3.66	4.8	4.21	3.67		
rel gene expression	0.079109787	0.035896824	0.054033577	0.078563336	**0.06**	**0.02**
	2A9	2A10	2A11	2A8		
B ACTIN	19.2	23.12	24.01	18.8		
Fpn	20.72	27.09	27.98	20.71		
dct (ctGOI-ctBACT)	1.52	3.97	3.97	1.91		
rel gene expression	0.348685917	0.063813258	0.063813258	0.266092546	**0.19**	**0.14**
					Mean	**sd**
Fold change in alcohols over controls	4.407620464	1.777685362	1.180992661	3.386981249	**2.69**	**1.5**

Table A12: Fold change of ferroportin mRNA in alcohol mice over control mice by RT-PCR (4 weeks batch)

	4C9	4C6	4C10	4C8	Mean	sd
B ACTIN	24.15	20.97	24.01	21.77		
Fpn	27.45	23.43	27.54	24.43		
dct (ctGOI-ctBACT)	3.3	2.46	3.53	2.66		
rel gene expression	0.10153155	0.181746565	0.086569342	0.158219574	**0.12**	**0.05**
		4A6		4A8		
B ACTIN	22.2	21.82	22.32	20.24		
Fpn	26.44	24.66	25.81	22.48		
dct (ctGOI-ctBACT)	4.24	2.84	3.49	2.24		
rel gene expression	0.052921582	0.139660892	0.089003137	0.211686328	**0.09**	**0.04**
					Mean	**sd**
Fold change in alcohols over controls	0.52123288	0.768437591	1.028113827	1.337927555	**0.91**	**0.4**

Table A13: Fold change of dcytb mRNA in alcohol mice over control mice by RT-PCR (1 weeks batch)

	1C9	1C6	1C7	1C8		
B ACTIN	23.36	18.52	17.95	16.88		
Dcytb	30.46	24.09	24.1	22.68		
dct (ctGOI-ctBACT)	7.1	5.57	6.15	5.8	**Mean**	**sd**
rel gene expression	0.00728932	0.021051	0.014082038	0.017948412	**0.02**	**0.01**
	1A9	1A6	1A7	1A8		
B ACTIN	23.13	17.58	17.84	17.37		
Dcytb	30.59	24.4	23.59	24		
dct (ctGOI-ctBACT)	7.46	6.82	5.75	6.63	**Mean**	**sd**
rel gene expression	0.00567958	0.008851	0.018581361	0.010096506	**0.01**	**0.01**

Fold change in alcohols over controls	0.77916458	0.420448	1.319507911	0.562529242	**0.77**	**0.39**

Table A14: Fold change of dcytb mRNA in alcohol mice over control mice by RT-PCR (2 weeks batch)

	2C9	2C10	2C11	2C8		
B ACTIN	22.08	23.11	21.53	20.18		
Dcytb	31.44	29.77	26.36	26.63		
dct (ctGOI-ctBACT)	9.36	6.66	4.83	6.45	**Mean**	**sd**
rel gene expression	0.00152181	0.009889	0.035158078	0.011438169	**0.01**	**0.01**
	2A9	2A10	2A11	2A8		
B ACTIN	22.55	22.52	24.01	18.8		
Dcytb	29.12	27.83	29.54	23.77		
dct (ctGOI-ctBACT)	6.57	5.31	5.53	4.97	**Mean**	**sd**
rel gene expression	0.01052526	0.025208	0.021642335	0.031906629	**0.02**	**0.01**
Fold change in alcohols over controls	6.91629785	2.549121	0.615572207	2.789487333	**3.22**	**2.65**

Table A15: Fold change of dcytb mRNA in alcohol mice over control mice by RT-PCR (4 weeks batch)

	4C9	4C6	4C10	4C8		
B ACTIN	24.15	20.97	24.01	21.77		
Dcytb	30.72	25.85	30.65	26.91		
dct (ctGOI-ctBACT)	6.57	4.88	6.64	5.14	**Mean**	**sd**
rel gene expression	0.0105252 6	0.03396	0.01002676 5	0.02835 9974	**0.02**	**0.0 1**
	4A9	4A6	4A10	4A8		
B ACTIN	22.2	21.82	22.32	20.24		
Dcytb	30.21	25.83	28.97	26.66		
dct (ctGOI-ctBACT)	8.01	4.01	6.65	6.42	**Mean**	**sd**
rel gene expression	0.0038792 7	0.06206 8	0.00995750 5	0.01167 851	**0.02**	**0.0 3**
Fold change in alcohols over controls	0.3685673	1.82766 3	0.99309249 5	0.41179 5509	**0.90**	**0.6 8**

Table A16: Fold change of hephaestin mRNA in alcohol mice over control mice by RT-PCR (1 weeks batch)

	1C9	1C6	1C7	1C8		
B ACTIN	23.36	18.52	17.95	16.88		
Hephestin	26.44	22.92	21.93	20.28		
GOI-BACT	3.08	4.4	3.98	3.4	**Mean**	**Sd**
EXPRESSION	0.118257	0.047366	0.063372467	0.094732285	**0.08**	**0.03**
	1A9	1A6	1A7	1A8		
B ACTIN	23.13	17.58	17.84	17.37		
Hephestin	26.59	21.48	22.16	21.74		
GOI-BACT	3.46	3.9	4.32	4.37	**Mean**	**sd**
EXPRESSION	0.090873	0.066986	0.050066867	0.048361406	**0.06**	**0.02**

Table A17: Fold change of hephaestin mRNA in alcohol mice over control mice by RT-PCR (2 weeks batch)

	2C9	2C10	2C11	2C8		
B ACTIN	22.08	23.11	21.53	20.18		
Hephestin	25.44	27.42	26.21	23.91		
GOI-BACT	3.36	4.31	4.68	3.73	**Mean**	**sd**
EXPRESSION	0.097396	0.050415	0.03901033	0.075362989	**0.07**	**0.03**
	2A9	2A10	2A11	2A8		
B ACTIN	22.55	22.52	24.01	18.8		
Hephestin	27.61	26.65	27.72	21.42		
GOI-BACT	5.06	4.13	3.71	2.62	**Mean**	**sd**
EXPRESSION	0.029977	0.057114	0.076415017	0.162667732	**0.08**	**0.06**
RELATIVE EXP ALC OVR CONTROLS	0.307786	1.132884	1.958840595	2.158456473	**1.39**	**0.5**

Table A18: Fold change of hephaestin mRNA in alcohol mice over control mice by RT-PCR (4 weeks batch)

	4C9	4C6	4C10	4C8		
B ACTIN	24.15	20.97	24.01	21.77		
Hephestin	29.21	23.43	28.48	24.49		
GOI-BACT	5.06	2.46	4.47	2.72	**Mean**	**sd**
EXPRESSION	0.029977	0.181747	0.045122787	0.151774361	**0.10**	**0.08**
	4A9	4A6	4A10	4A8		
B ACTIN	22.2	21.82	22.32	20.24		
Hephestin	28.29	24.17	27.21	23.23		
GOI-BACT	6.09	2.35	4.89	2.99	**Mean**	**sd**
EXPRESSION	0.01468	0.196146	0.033725882	0.125869444	**0.09**	**0.08**
RELATIVE EXP ALC OVR CONTROLS	0.48971	1.079228	0.747424624	0.829319546	**0.79**	**0.24**

Table A19: Fold change in expression of DMT1 protein in alcohol mice over controls for the periods of 1, 2 and 4 weeks

		1Controls	1Alcohols	2Controls	2Alcohols	4Controls	4Alcohols
Batch7	DMT1	64	64	71	699	36	273
	B-ACTIN	248	60	282	674	235	287
	Normalized	0.258065	1.0666667	0.251773	1.037092	0.153191	0.95122
	Fold change		4.1333333		4.119154		6.20935
Batch8	DMT1	139	308	42	56	18	117
	B-ACTIN	1372	930	2089	836	1219	351
	Normalized	0.101312	0.3311828	0.020105	0.066986	0.014766	0.333333
	Fold change		3.268941		3.331738		22.57407
Batch9	DMT1	147	298	56	77	9	395
	B-ACTIN	1355	1099	2199	976	62	452
	Normalized	0.108487	0.2711556	0.025466	0.078893	0.145161	0.873894
	Fold change		2.4994274		3.097976		6.020157

Table A20: Fold change in expression of ferroportin protein in alcohol mice over controls for the periods of 1, 2 and 4 weeks

Batch9	Weeks	1Controls	1Alcohols	2Controls	2Alcohols	4Controls	4Alcohols
	FPN	864	1500	636	1414	1361	1710
	b-actin	1750	1850	1472	2844	2559	2546
	normalized	0.493714	0.810811	0.432065	0.497187	0.531848	0.671642
	fold change in alcohols		1.642267		1.150722		1.262844
Batch 8	FPN	73	63	58	198	170	183
	b-actin	685	753	997	1332	1162	1426
	normalized	0.106569	0.083665	0.058175	0.148649	0.146299	0.128331
	fold change in alcohols		0.785079		2.555219		0.87718
Batch 7	FPN	522	434	254	285	340	303
	b-actin	1691	1720	2266	2792	1998	2033
	normalized	0.308693	0.252326	0.112092	0.102077	0.17017	0.149041
	fold change in alcohols		0.8174		0.910659		0.875834

Figure A1:Standard curve for serum iron estimation

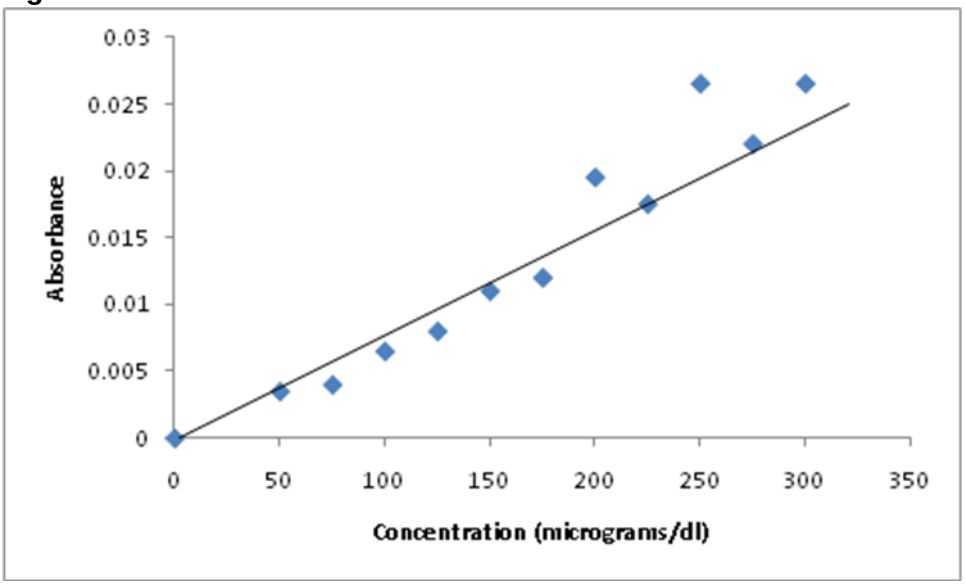

Table A21: Data showing serum iron in alcohol and control mice for the periods of 1, 2 and 4weeks. Serum iron in micrograms/dl

	1 week		2 week		4 week	
	control	alcohol	control	alcohol	control	alcohol
Batch 5	150.3	129.3	58.5	66.1	117.168	112.158
Batch 6	196	139.7	97.643	114.549	117.168	102.14
Batch 7	139.7	129.3	108.914	114.549	92.122	182.286
Batch 8	74.1	129.3	148.361	114.549	157.24	192.304

Figure A2: Plate arrangement for RT-PCR for DMT1, ferroportin, dcytb and hephaestin