AMERICAN UNIVERSITY OF BEIRUT

EFFECT OF COPPER CHELATORS ON FERROXIDASE AND IRON BINDING PROTEINS: IMPLICATIONS ON WILSON DISEASE

by
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How easy it seems to me now, spending hours in a white gown and powdered gloves. How easy seeking knowledge and truth in a nation with no impediments. How easy filling pages with evidences and actualities. And yet how hard, landing on words capable of conveying my gratitude.

I want to freely say

Thank you, God, my rock, and my stronghold. It is through the power of your blessings that I was ever able to accomplish my goals.

Thank you, my dearest parents, my guardians, and my angels. The walls of my room testify your attentive inquiry of my every detail. My heart is filled with gratitude and passion. It is filled with the love and wisdom that you have delicately poured into.

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AN ABSTRACT OF THE THESIS OF

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Title: Effect of Copper Chelators on Ferroxidase and Iron Binding Proteins: Implications on Wilson Disease

Copper is a micronutrient that serves important structural and catalytic roles in many proteins and enzymes. However, because of its redox potential, uncontrolled levels may be deleterious to the cell. Ceruloplasmin (Cp) belongs to the multi copper oxidase family of proteins that play a significant role in iron homeostasis particularly in Fe loading into serum transferrin. Defective copper loading into apoceruloplasmin may result from mutations in the ATP7B gene characterizing Wilson Disease (WD). Patients with WD are treated with copper chelators. Since copper mediates Cp ferroxidase activity, we postulate that in WD patients copper chelators will decrease level of copper incorporation into Cp, which would affect its ferroxidase activity and consequently disturb iron homeostasis. Therefore, our study aimed at investigating the *in vitro* effects of copper chelators, Penicillamine (PA) and Bathocuproine Disulfonate (BCS), on: viability of HepG2 cells (MTT assay); Cp ferroxidase activity (P-Phenylenediamine [pPD] oxidase assay); mitochondrial depolarization (Mito-JC1); ROS generation (NBT reduction); Electron transport chain (ETC) enzyme activities; and expression of Cp, ferritin, and transferrin (Western Blotting). We further examined serum iron level, Cp ferroxidase activity and expression of iron binding proteins in WD patients treated with PA.

Copper reduced the viability of HepG2 cells in a dose but not time dependent manner (EC_{50} = 50μM). Cu induced mitochondrial membrane depolarization and reduced ETC enzymes (I & II) activities without generating ROS. HepG2 cells co-treated with Cu and BCS (50 μM: 250 μM) showed restoration of cell viability while maintaining mitochondrial membrane depolarization. However, co-treatment of HepG2 cells with Cu and PA (50 μM: 250 μM) altered cell morphology, increased cytotoxicity resulting in further decrease in viability, increased ROS generation, depolarized mitochondrial membrane, inhibited ETC enzyme activities, and induced cell cycle arrest at G2/M.

Variation in the expression of Cu and Fe binding proteins in Cu and /or Cu chelator treated HepG2 cells were also examined. Our findings showed: a) increased Cp levels with Cu, while it decreased with PA or BCS; b) a similar profile to Cp was observed with transferrin; c) ferritin expression decreased with Cu, and Cu-PA, while it increased with

BCS treated cells. A significant reduction in pPD oxidase activity of Cp was obtained in HepG2 cells treated with Cu chelators alone or co-treated with Cu-PA/BCS.

The effect of copper chelator, PA, on Fe homeostasis was further examined in 5 PA treated WD patients, from two Lebanese families, who are homozygous for a disease causing mutation in the ATP7B gene. All patients had very low serum Cp ferroxidase activity (0.007-0.01 units) compared to control values (0.022-0.027 units). A mild elevation in serum ferritin levels was noted in 2 patients while transferrin levels were in the normal range.

To sum up, ferroxidase activity in both HepG2 cells and serum of WD patients was significantly decreased in Cp oxidase activity. This would influence Fe incorporation into transferrin that may consequently lead to iron deposition in the liver. The implication and significance of our findings necessitate reevaluation of PA treated WD patients for iron complications.

CONTENTS

ILLUSTRATIONS

TABLES

CHAPTER I

INTRODUCTION

American neurologist, Samuel Alexander Kinear Wilson initially explained **Wilson Disease (WD)** in 1912 as hepatolenticular degeneration. He described a familial case with neurological manifestations and liver cirrhosis. Cummings identified involvement of copper in 1948 (Gitlin 2003) and it wasn't until 1993, that the ATP7B gene was cloned and recognized as encoding a Cu-transporting P-type Adenosine triphosphatase (ATPase) involved in excretion of copper (Lin, et al. 2010).

WD is an inherited autosomal recessive disorder characterized by failure of Cu incorporation into Ceruloplasmin (Cp) and decreased biliary copper excretion. As a consequence, Cu accumulates in various organs including the liver, brain, cornea, and the kidney, leading to chronic liver disease and/or neurological impairment (Roberts and Cox 1998).

Diagnosis of WD is based on laboratory tests, clinical symptoms, and genetic screening. Important diagnostic laboratory tests include: High 24 hr urinary copper excretion (>100μg/dl), low Serum Ceruloplasmin (<20 mg/dl), and high hepatic copper content (> 250μg/g dry tissue) (Sternlieb 1993).

Clinical presentation however may vary between patients with:

- Hepatic disorders: Range from asymptomatic transaminasemia, acute or chronic hepatitis, to fulminant hepatic failure and cirrhosis (Ferenci, et al. 2003).
- Neurological symptoms: Mild tremors, articulating and writing problems are early

symptoms, which may progress into dysarthria, Parkinson-like features, and seizures. Some WD patients with neurological manifestations may encounter psychiatric abnormalities like depression, personality changes, psychosis, and irritability (Oder, et al. 1991).

- Mixed presentation: Patients manifest both neurological and hepatic symptoms.

In addition, some patients have ophthalmologic involvement. Kayser-Fleischer (KF) rings reflect deposition of Cu in the Descemet's membrane, visible as a greenish-yellow or brown ring at the limbus of the cornea, and detected by slit-lamp examination (Krajacic, et al. 2006).

Age of onset in WD patients is highly variable (Ferenci, et al. 2007). Signs of WD are rarely observed in children before the age of 3. Hepatic disorder is the most common feature in children between the ages of 10 and 13, a decade earlier than patients with neurological manifestations (Gitlin 2003).

Genotypic analysis is the best diagnostic tool especially in asymptomatic patients. The most common mutations in Central, North, East Europe, and East Asia are H1069Q, 3400delC, 2299insC, and R778L respectively (Dastsooz, et al. 2013; Ferenci 2006), the majority of which are compound heterozygous. In Lebanon and the Arab world, consanguinity is very common, increasing the chance of having WD patients who are homozygous for the mutations (Barada, et al. 2010).

A. P- type ATPases

There are four major classes of ATPases that function in transporting ions or molecules into and out of cells or organelles within cells. These classes are the F_0F_1 (F), V, P , and ABC ATPases. What distinguishes the P- type from the rest is its ability to form a phosphorylated intermediate state during the transport cycle (Pedersen 1987). Both V and F types are similar in structure and transport H^+ ions. However, while F type ATPases are found in bacterial and mitochondrial membranes and are mainly involved in ATP synthesis in aerobic organisms, V type ATPases are present in vacuoles, lysosomes, synaptic vesicles, and Golgi complexes, and transport H^+ at the expense of ATP hydrolysis (Finbow and Harrison 1997). ABC (ATP- Binding Cassette) ATPases are found in all species and transport almost any type of molecule from large polypeptides to small ions (Saurin, et al. 1999).

The P- type ATPases are a large family of integral membrane proteins and are divided into P_I ATPases that transport transition metals or metalloid cations such as Cu, Cd, and Zn, and P_{II} ATPases that transport alkali metal cations. The latter includes Ca^{+2}-ATPases, involved in regulation of Ca^{+2} and is divided into two main classes in mammals: Sarco(endo)plasmic (SERCA) and plasma membrane (PMCA) Ca^{+2} ATPases; Na^+,K^+-ATPases, involved in maintaining resting potential, Na^+ dependent nutrient uptake, and fluid transport by the kidney tubules; H^+,K^+-ATPases, involved in H^+ and K^+ secretion in the stomach (Moller, et al. 1996).

Human Cu-ATPases, ATP 7A and ATP 7B, belong to P_I type of ATPases found in the membrane of the Trans Golgi Network, in endocytic vesicles, and sometimes at the plasma membrane (Suzuki and Gitlin 1999). Under normal physiological conditions, ATP7A and ATP 7B rotate between these locations in response to copper levels, accordingly Cu is

incorporated into copper-dependent enzymes, stored in vesicles, or released extracellularly for export (Figure 1) (La Fontaine and Mercer 2007).

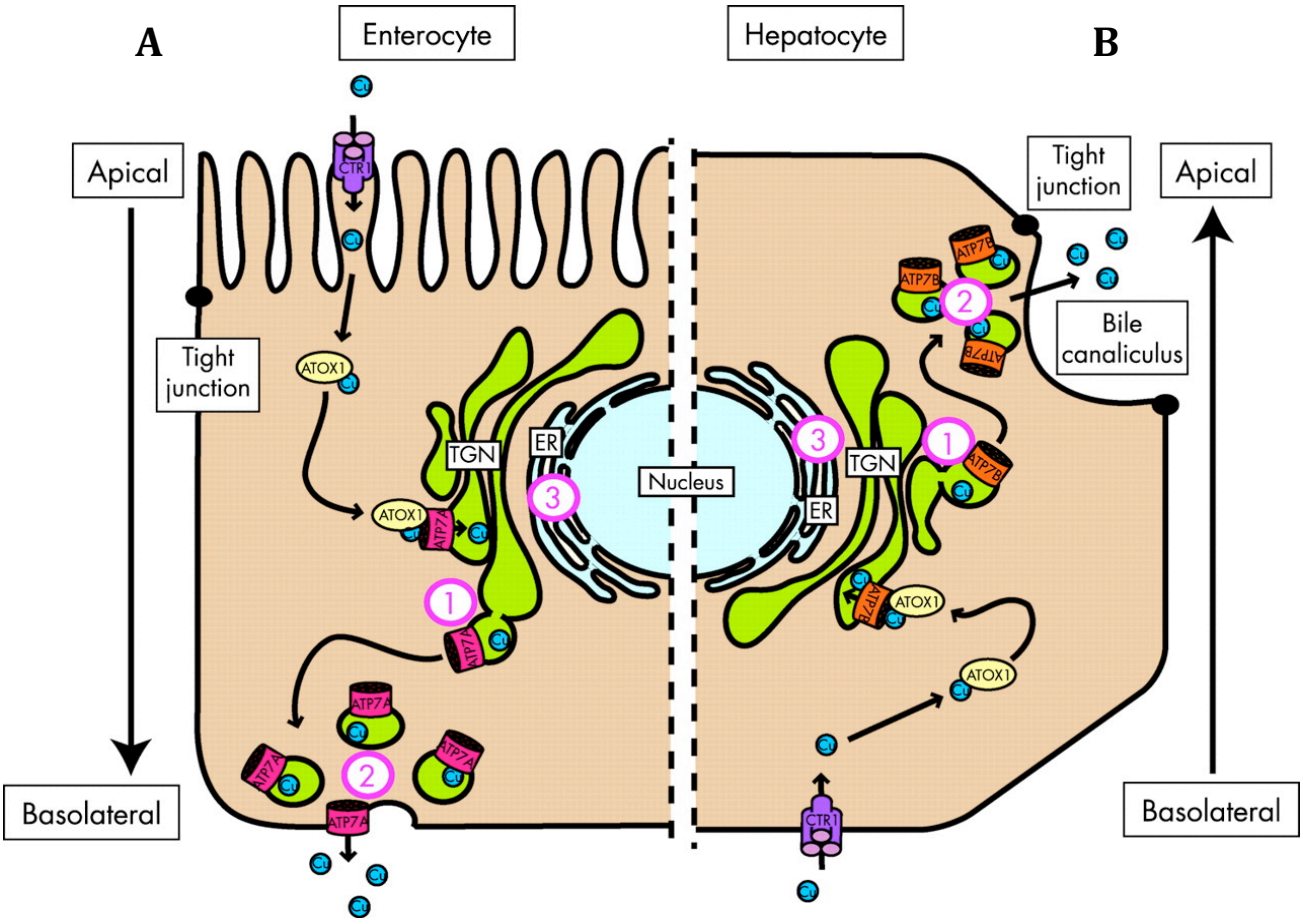

Figure 1: Copper homeostasis at the (A) enterocyte and (B) hepatocyte (de Bie, et al. 2007)

Cu-ATPases are characterized by eight trans-membrane segments, six regulatory heavy metal-binding domains (MBDs) at the N terminus, two intracellular large loops comprising the A-domain (phosphatase), and the P and N domains (phosphorylation and ATP-binding). Highly conserved motifs include the DKTG sequence carrying the Aspartate residue that is phosphorylated in the reaction cycle; the TGES sequence prior to the phosphorylation site, and a GDGXNDXP motif after the ATP-binding domain (Portmann and Solioz 2005).

1. ATP7A

ATP7A, located on chromosome Xq13.2-13.3, encompasses 23 exons, and is expressed in all cells and tissues except the liver (Chelly, et al. 1993). Under normal conditions, ATOX1, a copper chaperone, donates Cu to ATP7A, located at the trans golgi network, to be translocated across intracellular membranes into the secretory pathway. There Cu is incorporated to Cu-dependent enzymes. A pathogenic mutation in the gene coding for ATP7A leads to failure of Cu incorporation onto these enzymes and consequently dysfunction in several biochemical pathways leading to clinical manifestations that are collectively known as Menkes Disease (MD) (Mercer, et al. 1993).

MD, an X-linked inherited recessive disorder is characterized by neurodegenaration, mental and growth retardation, connective tissue abnormalities, and “kinky” hair, and the diagnosis is supported by low serum Ceruloplasmin and Cu (Tumer and Klomp 2011). Plasma catecholamine analysis may be performed, since it is indicative of dopamine β hydroxylase deficiency, in neonates who normally have low serum Cp and Cu (Kaler, et al. 2008). MD patients are treated with parenteral copper-histidine supplementation that shows

positive results in patients who have partially functional ATP7A (Kreuder, et al. 1993).

2. ATP7B

ATP7B is located on chromosome 13q14.3 and has 21 exons. The 165 KDa protein contains 8 transmembrane spanning domains, 6 N terminal Cu-binding sites, a transmembrane cation channel, and an ATP-binding domain toward the carboxy terminus (Figure 2) (Bull, et al. 1993). It is expressed in the liver, kidney, placenta, and to a lesser extent in the heart, brain, lung, and muscle. A defect in the gene coding for ATP7B leads to Wilson Disease (WD).

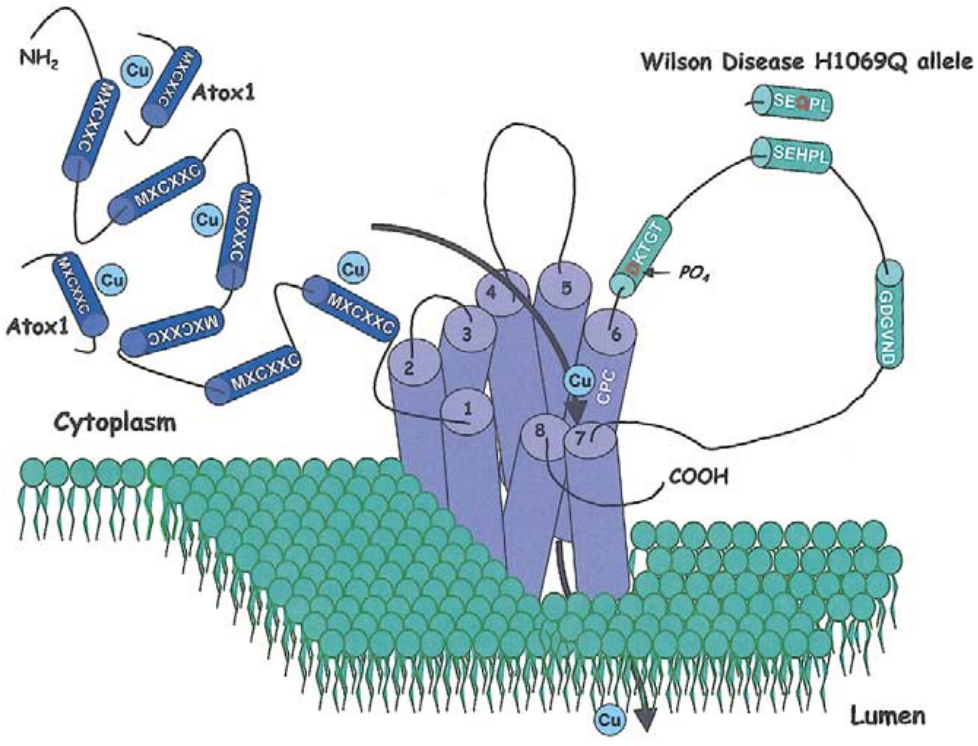

Figure 2: Structure of ATP7B (Gitlin 2003)

Cu is delivered from ATP7B to apo-Ceruloplasmin in the secretory pathway. Terada et al confirmed this by introducing the cDNA of ATP7B to *Long-Evans Cinnamon* (LEC) rats, animal models of WD, lacking functional ATP7B, and producing only apo-Cp. Secretion of holo-Cp, the Cu bound and oxidase active form, demonstrated that Cu loading onto Cp was restored in the liver of these rats (Terada, et al. 1998). Two other established animal models of WD are the ATP7B-/- mice and the toxic milk mice, all of which have hepatic Cu accumulation (Merle, et al. 2010a).

B. Copper in biological systems

The main source of human exposure to copper is diet, abundant in nuts, seeds, seafood and liver (Sandstead 1995). In adults, average copper intake varies from 0.6 to 1.6 mg/day. Copper is fundamental to many biological systems that control growth and development, more specifically as catalysts and enzymes including superoxide dismutase, cytochrome-c oxidase, tyrosinase, lysyl oxidase, dopamine-β-monooxygenase, monophenol monooxygenase, phenylalanine hydroxylase, and Ceruloplasmin (Table 1) (Linder and Hazegh-Azam 1996). Nevertheless, Cu is detrimental if found in excess. Hence mechanisms that control and balance the level of Cu within strict levels are necessary for Cu homeostasis.

Absorption of copper depends on its chemical form. Cu ions can exist in both an oxidized (Cu^{+2}) cupric, or reduced (Cu^{+}) cuprous state. While the solubility of Cu^{+} is in the sub-micromolar range, Cu^{+2} is fairly soluble. Most of the ingested Cu is in the Cu^{+2} state - since in the presence of oxygen, Cu^{+} is easily oxidized to Cu^{+2} (Linder 1991). Copper is absorbed across the mucosal cells lining the stomach and the small intestine by the human Cu

ENZYME	FUNCTION
Cytochrome-c oxidase	Electron transport in mitochondria
Cu/Zn- SOD	Free radical detoxification
Metallothionein	Storage of excess Cu and other divalent metal ions.
Ceruloplasmin	Ferroxidase, acute phase reactant, Cu transport
Lysyl oxidase	Cross linking of collagen and elastin
Tyrosinase	Formation of melanin
Dopamine-β-monooxygenase	Catecholamines production
Peptidylglycine monooxygenase	Bioactivation of peptide hormones
Hephaestin	Ferroxidase in enterocytes; aids iron absorption

Table 1: Copper dependent enzymes in mammals (Tapiero, et al. 2003)

transport protein 1(hCTR1) with the help of reductases, Steap2 and Dcytb, which reduce $Cu+^{2}$ to Cu^{+}. Once in the cytoplasm, Cu is bound to Metallothionein (MT) or destined for secretion by binding to Cu chaperons like Atox 1 (de Romana, et al. 2011).

Copper is secreted into the portal circulation and transported to the liver bound to serum albumin, low molecular weight Cu-Histidine complexes, and transcuprein, a copper binding protein with high affinity to copper (de Romana et al., 2011). Part of the endogenous pool of Cu is replenished by the reabsorption of this metal from digestive juices, such as the saliva, bile, gastric, and pancreatic juices (Linder and Hazegh-Azam 1996). The biliary tract is the only mechanism for physiological copper excretion in bile, the amount of which is proportional to the size of copper pool in the liver (Gitlin, et al. 1960).

Upon reaching the liver, uptake of Cu occurs by the receptor **CTR1**. Copper however, because of its high redox potential, never exists as a free ion intracellularly, as it contributes to free radicals generation that damage proteins, lipids, and nucleic acids (Halliwell and Gutteridge 1990). Once inside the cytoplasm, copper is delivered to different compartments within the cell by the help of specific cytosolic metallochaperones, such as Atox1, CCS, COX 17, and COMMD 1 (Figure 3).

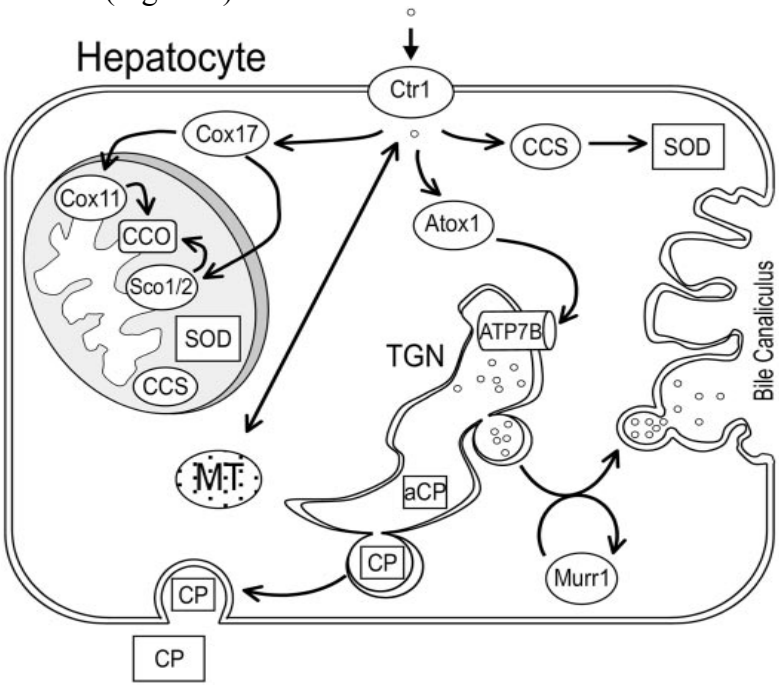

Figure 3: Copper transport in hepatocytes (Prohaska and Gybina 2004)

Atox 1 or HAH1, is the human homologue of Atx 1 (anti-oxidant 1) in yeast. It mediates transfer of Cu to the trans golgi network where Cu gets incorporated into The P-type ATPase ATP7B (Larin, et al. 1999). The copper-binding motif MXCXXC is found on both Atox 1 and ATP7B. The direct interaction of Atox 1 and ATP7B with each other depends on the availability of copper (Portnoy, et al. 1999). When Cu levels are low, ATP7B is localized in the trans golgi network. When Cu levels are high, ATP7B translocates to intracellular vesicles, excreting Cu into the bile (Schaefer, et al. 1999).

The copper chaperone protein **CCS**, mediates the transfer of Cu to the Cu/Zn superoxide dismutase (SOD) required in the cytoplasm to prevent the harmful effects of radicals (Culotta, et al. 1997). It is significant to mention that in cases of low levels of Cu, Cu/Zn-SOD loses its activity (Levy, et al. 2001).

COX 17 shuttles Cu from CTR1 into the mitochondrial membrane space (Glerum, et al. 1996). **COX 11** is a mitochondrial membrane bound protein that heterodimerizes with COX 17 and mediates copper transfer to Cytochrome-c oxidase which is the terminal oxidase in the aerobic respiration that reduces Oxygen to water (Ferguson-Miller and Babcock 1996).

Copper **m**etabolism **M**URR1 **D**omain protein **1** (**COMMD1**), is a copper chaperone involved in hepatic copper excretion mediated by ATP7B from the trans golgi to the bile canaliculus (Burstein, et al. 2005).

C. Ceruloplasmin

Holmberg and Laurell were the first to isolate Ceruloplasmin (Cp) from the plasma and identify it as a copper containing protein (Holmberg and Laurell 1948). Ceruloplasmin, an abundant protein, is a member of the multicopper oxidase family of enzymes, found in the plasma of all vertebrates. Cp is also a prototype of "moonlighting" proteins whose function changes according to substrate, differential localization, and expression, which allows Cp to meet the physiological changes and pathological conditions of the organism (Marques, et al. 2012). Cp is an acute phase reactant protein. The serum concentration of Cp increases during inflammation or trauma, as a consequence to the inflammatory cytokines that mediate gene transcription in hepatocytes (Gitlin 1988). Moreover, Ceruloplasmin levels increase three to fourfold during pregnancy, since estrogen increases synthesis of proteins in hepatocytes (Laurell, et al. 1968).

Cp is predominantly synthesized in the liver. Nevertheless, Cp gene expression has been shown in various other tissues including the spleen, lungs, testis, and brain (Aldred, et al. 1987). Cp has a half life of 5 and a half days in plasma, and studies reveal that copper bound to Cp is not exchanged after Cp is synthesized (Sternlieb, et al. 1961).

Cp can be expressed either as a secreted form, the majority of which is synthesized in the liver, or anchored to the cell membrane. Glycosylphosphatidylinositol (GPI)- Cp is an isoform of Cp highly expressed in the central nervous system (Patel, et al. 2000). GPI-Cp is generated by the alternative splicing of exons 19 and 20. A study conducted by Marques et al., shows that GPI-Cp isoforms are not only expressed in the brain, but also on cell membranes of immune and hepatic cells (Marques, et al. 2012). Ceruloplasmin is first synthesized as

apo-ceruloplasmin. Copper loading into apo-Cp occurs in the trans golgi network, giving rise to holoceruloplasmin, the active form (Figure 4). In WD patients this loading is impaired resulting in the secretion of apoCp that is highly unstable and rapidly degraded in the serum (Terada, et al. 1998).

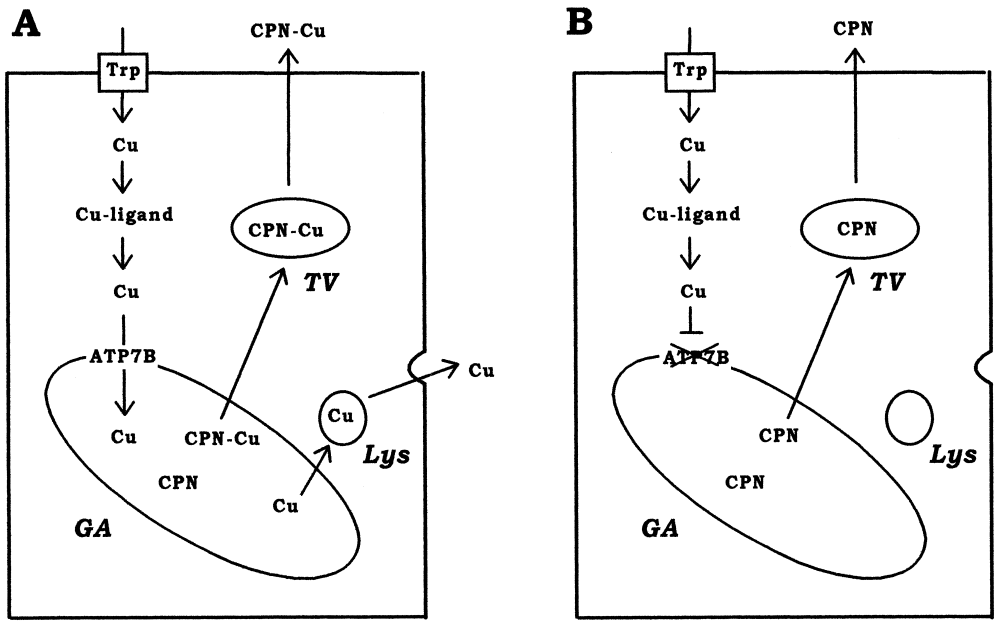

Figure 4: Normal Cu transport (A), and Cu transport in WD (B). (Terada, et al. 1998)

Cp is a monomeric glycoprotein, composed of three 42-45 KDa homologous domains, giving it an approximate size of 132KDa. The serum clotting factors V and VIII and Cp were shown to be structurally similar proteins sharing common nucleotide and amino acid sequences (Church, et al. 1984). Human Cp is encoded in 20 exons located on chromosome 3q23-q24 (Daimon, et al. 1995).

Cp contains three type I copper sites, a single type II, and two type III copper sites arranged in a triangular array of 6 cupredoxin-type domains (Figure 5) (Bento, et al. 2007; Calabrese, et al. 1989). Type I Cu ions are arranged in 3 mononuclear sites (in domains 2,4, and 6) and are coordinated to a cysteine and two histidine residues (Bento, et al. 2007). Cp is often referred to as the blue protein, since transfer of charges at sites I, between Sulfur (on Cysteine) and copper, results in strong absorption at 600nm (Hellman and Gitlin 2002).

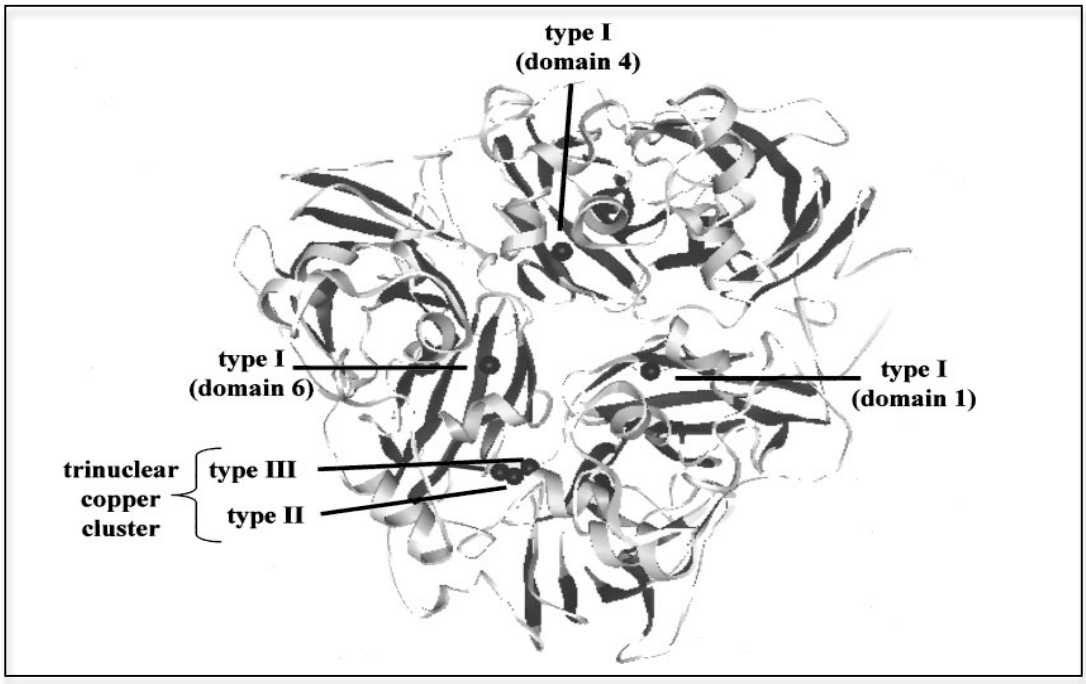

Figure 5: Structural model of human Ceruloplasmin (Hellman and Gitlin 2002)

Type II and III Cu ions form a trinuclear cluster at the interface of domains 1 and 2, to which oxygen binds during the catalytic cycle (Figure 6) (Calabrese, et al. 1989). One of the three T1 sites is permanently reduced, while the other two are redox active (Quintanar, et al. 2004). Ferrous ions bind these sites found in domains 4 and 6. Iron binding sites are composed of 2 glutamate, one aspartate, and one histidine residue, in addition to two water molecules (Vashchenko and MacGillivray 2013). Site-directed mutagenesis in residues E935 and H940A of domain 6, reduced ferroxidase activity by 50% confirming the involvement of these residues in iron binding (Quintanar, et al. 2004). Electrons are passed to the type I copper from the substrate, which are then transferred to the trinuclear cluster and finally to the oxygen molecule found at this site (Messerschmidt, et al. 1989). Multicopper oxidases share the same amino acid sequences of the copper ligands, however, the substrates are different, as well as the number of Type I coppers vary from protein to protein (Machonkin, et al. 2001).

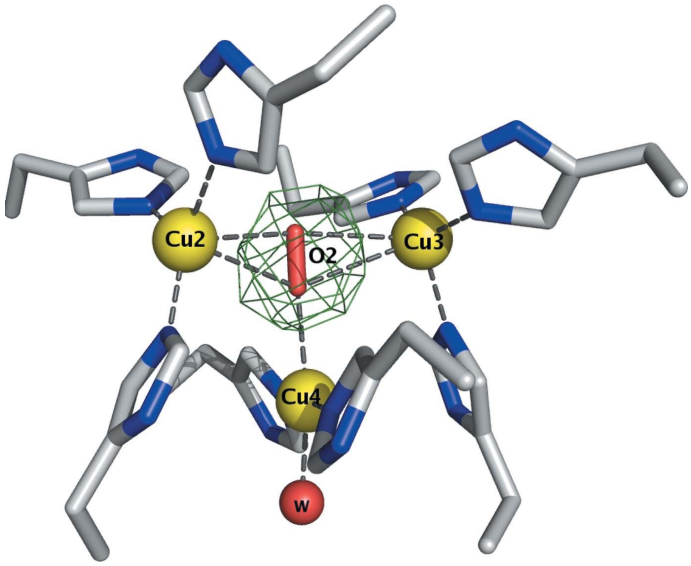

Figure 6: The trinuclear cluster between domains 1 and 6 (Bento, et al. 2007)

Cp catalyzed ferroxidase reaction is given by the following equation:

$4Fe^{+2} + 4H^{+} + O_2 \rightarrow 4Fe^{+3} + 2H_2O$ (Taylor, et al. 2005)

Metabolic labeling experiments showed that the six copper binding sites should be occupied without apparent hierarchy, to achieve the final state of Cp. Other investigators reported that it is possible to have partially metallated forms of Cp (Sedlak and Wittung-Stafshede 2007).

In WD patients, defective Cu incorporation into Ceruloplasmin leads to low serum ferroxidase activity (Merle, et al. 2010a). Measuring serum Cp is a useful diagnostic test in WD; however, it has been shown that 35% of hepatic WD patients have normal Cp levels (Merle, et al. 2010b). The immunological method of determining serum Cp recognizes both apoCp and holoCp. The enzymatic activity of Cp can be determined by its oxidase activity on diamines such as p-phenylenediamine (pPD) (Merle, et al. 2009). pPD was shown to be the best of a number of substrates used (White, et al. 2012), with Cp oxidase activity using pPD being superior to the immunological method (Macintyre, et al. 2004).

1. Aceruloplasminemia

Absence of the active form of Ceruloplasmin, due to a homozygous mutation of the Cp gene, is known as Aceruloplasminemia. It is a rare autosomal disorder characterized by neurodegeneration (Yoshida, et al. 1995). Since Cp does not cross the blood-brain barrier, there must be Cp synthesized within the CNS. Studies have shown that astrocytes synthesize Cp in the CNS, primarily in its GPI-Cp isoform, which is important for iron homeostasis (Klomp, et al. 1996). Absence of Cp results in accumulation of iron in the central nervous

system; however, it does not affect the metabolism of copper (Nittis and Gitlin 2002). Laboratory studies show microcytic anemia, complete loss of Cp ferroxidase activity, and high levels of ferritin in the serum of patients (Gitlin 1998). MRI of the brain shows deposition of iron in the basal ganglia, thalamus, striatum, dentate nucleus, as well as the liver (Miyajima, et al. 1998). Patients develop retinal degeneration, diabetes mellitus, and present with neurological features including dementia, dysarthria, and dystonia usually in the fourth or fifth decade of life (Logan, et al. 1994).

2. Hephaestin and Zyklopen

Hephaestin (Hp) and Zyklopen (ZP) are multicopper ferroxidases involved in exporting iron. Hp is an integral transmembrane protein expressed in the enterocytes and required for dietary iron export (Frazer, et al. 2001). Hp is mutated in the sex-linked anemia (*sla*) mice that develop severe to moderate microcytic anemia. Iron remains in the enterocytes not being able to be exported into circulation, leading to its loss by the exfoliated enterocytes, and hence anemia (Vulpe, et al. 1999).

Pregnant rats with dietary Cu deficiency produced offspring that were iron deficient (Pyatskowit and Prohaska 2008). Chen et al, first detected a multi-copper oxidase in BeWo cells (human placental cell line) that was thought to be involved in iron efflux. The protein was expressed in the placenta and mammary glands and had different expression patterns from Hephaestin and Ceruloplasmin. Chen et al called this protein Zyklopen (ZP). ZP protein is structurally similar to Cp (61.5% similarity) and Hp (66.2% similarity) (Chen, et al. 2010).

Although serum Cp levels in most WD patients is lowered, its structure is not altered. Moreover, many WD patients have normal Cp levels, while many normal individuals

unrelated to WD patients have low Cp levels (Holtzman, et al. 1967).

LEC rats contract hepatitis and eventually hepatocellular carcinoma due to abnormal Cu accumulation in the liver, similar to patients with Wilson Disease. Interestingly, an iron deficient diet in LEC rats prevented the development of hepatitis and cancer (Kato, et al. 1996).

The specific role of Cp and its analogues in iron metabolism is not well understood, but it has been demonstrated that Cp facilitates both cellular iron uptake (Attieh, et al. 1999) and release (Harris, et al. 1999).

D. Iron

Iron is one of the most abundant transition metals. It exists in the ferric (Fe^{+3}) form, which has low solubility and bioavailability (Wardman and Candeias 1996). Two other forms of iron exist in the diet: Inorganic, water insoluble, non-haem iron complexes found in vegetables, explaining thus the high prevalence of iron deficiency in vegetarian population (Zimmermann and Hurrell 2007), and haem iron proteins found in red meat, poultry, and fish which is efficiently absorbed (Fairweather-Tait 2004).

Iron is found in the body bound to several functional proteins and enzymes required for various biological processes. Such as, myoglobin, in the skeletal and cardiac muscles, cytochromes within the mitochondria, as well as catalase and cytochrome P450, indispensable for intracellular oxygen transport, breakdown of hydrogen peroxide, and drug detoxification respectively. Moreover, iron is required by ribonucleotide reductase, for DNA synthesis (Wang and Pantopoulos 2011).

There are two means by which sufficient amounts of iron enter the human body:

through the placenta during fetal development; and through the walls of the small intestine following birth. After the infant uses up the maternal iron, diet becomes the only source of this element. The average adult human body holds 3 to 4 g of iron (Leong, et al. 2003).

The fact that iron can participate in oxidation/reduction reactions makes it very useful in many enzymatic reactions. Binding of iron to protoporphyrin, results in haem, which is the essential oxygen-binding molecule in vertebrates (Ponka 1999). Similar to Cu, iron is highly reactive and toxic and its overload leads to various disorders (table 2) such as:

1. Freidreich Ataxia: caused by defective frataxin, a mitochondrial protein, suggested to play a role in iron-sulfur assembly and iron transport (Alper and Narayanan 2003).

2. Hereditary Hemochromatosis: caused by an increase in absorption of iron in the duodenum. The most common cause is a mutation in the HFE gene, encoding a β2-microglobulin binding protein, where iron accumulates in the liver, pancreas, heart, joints and skin (Whittington and Kowdley 2002).

Secondary iron overload results from additional mechanisms that cause iron deposition in body tissues such as ineffective erythropoiesis (Sideroblastic anemia, thalassemia major), alcohol consumption, and chronic hepatitis C (Whittington and Kowdley 2002).

In the Fenton reaction, the weak oxidant hydrogen peroxide produced by the SOD reacts with Ferrous ion (Fe^{+2}) to produce hydroxyl radical ($HO^{\bullet}$) (Pierre and Fontecave 1999).

The Fenton reaction: $Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + OH^{\bullet} + OH^-$

These Reactive Oxygen Species (ROS) will oxidize lipids, proteins, and nucleic acids, leading to cancer or cell death (Taketani 2005).

Protein	Function	Disorder
DMT1	Ferrous iron transporter	Iron deficiency anemia
Frataxin	Iron chaperone	Freidreich ataxia
Ferroportin	Ferrous iron exporter	Hemochromatosis type 4
Ceruloplasmin	Ferroxidase	Aceruloplasminemia
Transferrin	Plasma iron transporter	Atransferrinemia
HFE	Regulator of iron homeostasis	Hemochromatosis type 1
Hepcidin	Regulator of iron homeostasis	Juvenile hemochromatosis

Table 2: Iron imbalance disorders (Vashchenko and MacGillivray 2013)

Iron homeostasis is maintained by mechanisms involving duodenal enterocytes where absorption of iron occurs, macrophages that recycle iron from erythrocytes, and hepatocytes where iron is stored or released. While excess Cu can be excreted through the bile, there is no known regulated process for the removal of excess iron (Wijmenga and Klomp 2004). Iron loss is mainly due to sloughing of epithelial cells into the fecal stream, menstruation, pregnancy, and sweat (Beutler 2006).

1. Import of Iron

At the brush border, DcytB (Duodenal Cytochrome B) reduces the dietary ferric iron by ascorbate into the ferrous state to be transported into the enterocyte via DMT1 (Divalent Metal Transporter 1) expressed on the membrane of duodenal enterocytes (Collins, 2012). DMT1 is not specific to iron, transporting other transition metals as well (Figure 7). Once in the cytosol of the enterocyte, iron is either stored, or exported.

Unlike import, export of iron is restricted to certain cells including duodenal hepatocytes, macrophages, placental trophoblasts, hepatocytes, erythrocytes, neurons, and β cells of the pancreas (Vashchenko and MacGillivray 2013). Mobilization of iron from tissues for transport to other parts of the body is an important part of iron homeostasis (White, et al. 2012).

2. Export of Iron

Hepcidin, a peptide hormone produced by hepatocytes, regulates iron homeostasis by controlling its absorption and the release of recycled iron (Nemeth and Ganz 2009). Most forms of Hereditary Hemochromatosis result from Hepcidin deficiency. Hepcidin binds to the iron exporter Ferroportin (Fpn) on the surface of cells leading to the internalization and degradation of Fpn, and consequently decreasing iron release into the blood (Nemeth, et al. 2004). Deletion of the gene HAMP1 coding for Hepcidin, led to iron overload in mice (Nicolas, et al. 2001). Fpn is the only identified mammalian iron exporter, and is expressed on all cells involved in transferring iron to plasma, such as the absorptive cells of the intestine and tissue macrophages (Marques, et al. 2012). It is also known as Ireg 1 (Iron regulated gene 1) or Mtp1 (Metal Transporter Protein 1). Like DMT1, Fpn has 12 transmembrane

domains, and both termini in the cytoplasm (Liu and Theil 2005).

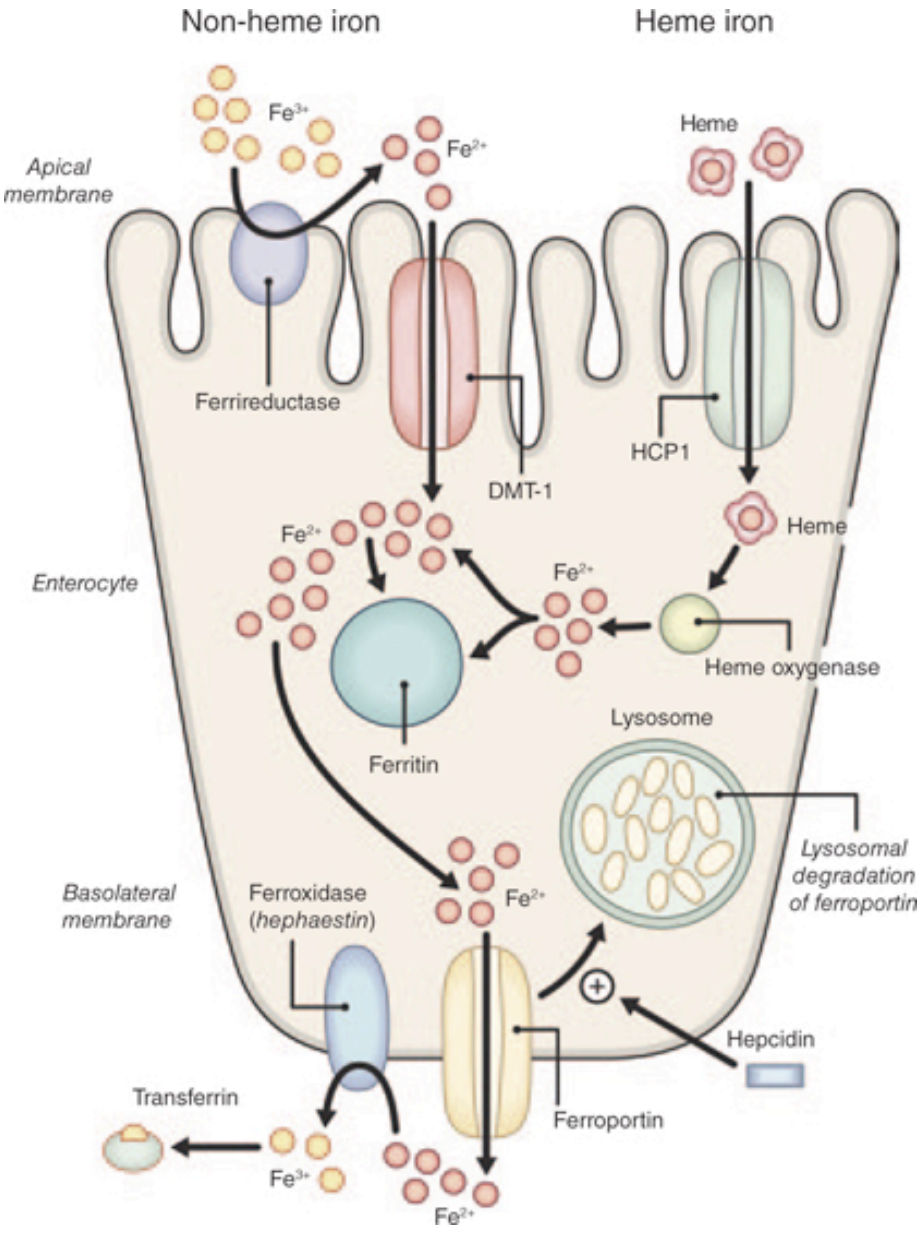

Figure 7: Mechanism of iron absorption (Rizvi and Schoen 2011)

Iron export depends on Hephaestin (in enterocytes) and Ceruloplasmin (in hepatocytes). These ferroxidases oxidize ferrous iron (Fe^{+2}) exported by Fpn into ferric iron Fe^{+3} in order to be incorporated into transferrin, the major iron transport protein (Harris, et al. 1999). Iron exported from macrophages is taken up by lactoferrin, which is secreted by activated inflammatory cells (White, et al. 2012). The role of Cu in iron transport is well established. ATP7B-/- mice showed a decreased Cp oxidase activity associated with a slight, but not significant, increase in liver iron stores (Merle, et al. 2010a).

3. Storage of Iron

Iron in the enterocytes, as well as in the hepatocytes once delivered there, are stored in the large hollow protein Ferritin. In mammals, most of this protein is produced by cytosolic ribosomes (Theil 2011). Iron is stored within ferritin as a hydrous ferric oxide nanoparticle, with a structure similar to ferrihydrite crystals (5Fe2-9H2O) (Liu and Theil 2005). Ferritin is composed of 24 subunits of 2 types: H and L. The H chain possesses ferroxidase activity and catalyzes oxidation of ferrous iron by oxygen producing H_2O_2, while L enhances mineralization of the ferric iron (Chasteen and Harrison 1999). Ferritin expression is regulated post translationally. Specific regions in the 3'UTR of the mRNA contain stem-loop structures, called Iron-Responsive Elements (IREs) required for iron dependant regulation. The IREs are recognized by IRPs - Iron Regulatory Proteins (Casey, et al. 1988). In iron deficient cells, IRP1 and IRP2, two closely related IRPs, bind to IREs in the 3'UTR mRNA of Ferritin, inhibiting translation of Ferritin (Torti and Torti 2002). Therefore Ferritin is an important marker that assesses iron depletion as well as overload.

4. Iron Transporter: Transferrin

Once ferric iron is exported bound to Transferrin (Tf) it is distributed and released in tissues mediated by transferrin Receptor (TfR1). Transferrin is an 80 Kda monomeric glycoprotein that can carry up to two iron ions. Tf has an important function in iron homeostasis through binding, sequestering, and transporting of ferric irons. Its concentration in normal serum is between 25 and 50 μmol/l. Only one third of it is occupied by iron to provide a buffering capacity in cases of acute increase in iron levels (Graham, et al. 2007). One of the fundamental roles of Tf is delivering iron for erythropoiesis, since genetic atransferrinemia in mice and humans lead to severe anemia (Bartnikas 2012).

The transferrin receptor (TfR1), expressed on all nucleated cells, aids in iron uptake, through a cycle of transferrin endocytosis/exocytosis. Tf becomes trapped within endocytic vesicles called endosomes. Iron binding to Tf is dependent on the pH of the milieu. The neutral pH of the plasma allows strong binding, whereas the acidity of the endosomes cause iron to be released from Tf (Dautry-Varsat, et al. 1983).

Each TfR binds one molecule of Tf and uptake of iron is proportional to the number of TfRs. Cells rich in iron are attributed with enhanced intracellular ferritin levels but lowered receptor numbers (Louache, et al. 1984). Similar to Ferritin, regulation of TfR gene expression is post translational, mediated by IRP1 and IRP2. In iron deficient cells, IRPs binds to IREs in the 3'UTR of TfR mRNA leading to an increase in cellular iron uptake (Mullner and Kuhn 1988).

5. Iron in the liver

The liver is the primary destination of iron after its absorption and it plays a major

role in iron homeostasis (figure 8). Hepatocytes recover iron from ferritin, hemopexin (circulating heme), haptoglobin (circulating hemoglobin) and lactoferrin. They have TfR1 and TfR2 receptors and are also capable of non-transferrin bound iron uptake through DMT1 (Garrick and Garrick 2009). Transferrin binds to TfR1 and/or TfR2 and is endocytosed into an endosome where iron is released by acidic pH. The gene STEAP 3 (Six Transmembrane Epithelial Antigen of the Prostate 3) is highly expressed in the liver. Its protein product co-localizes with TfR and DMT1 in endosomes and has ferrireductase activity, reducing Fe^{+3} to Fe^{+2}. Iron is then transferred to the "transit pool" from where it is either stored in ferritin, or distributed to iron-containing moieties such as haem or iron-sulfur clusters. Fe^{+2} is exported out of the liver through Fpn and oxidized by Cp before its incorporation into apotransferrin (Graham, et al. 2007).

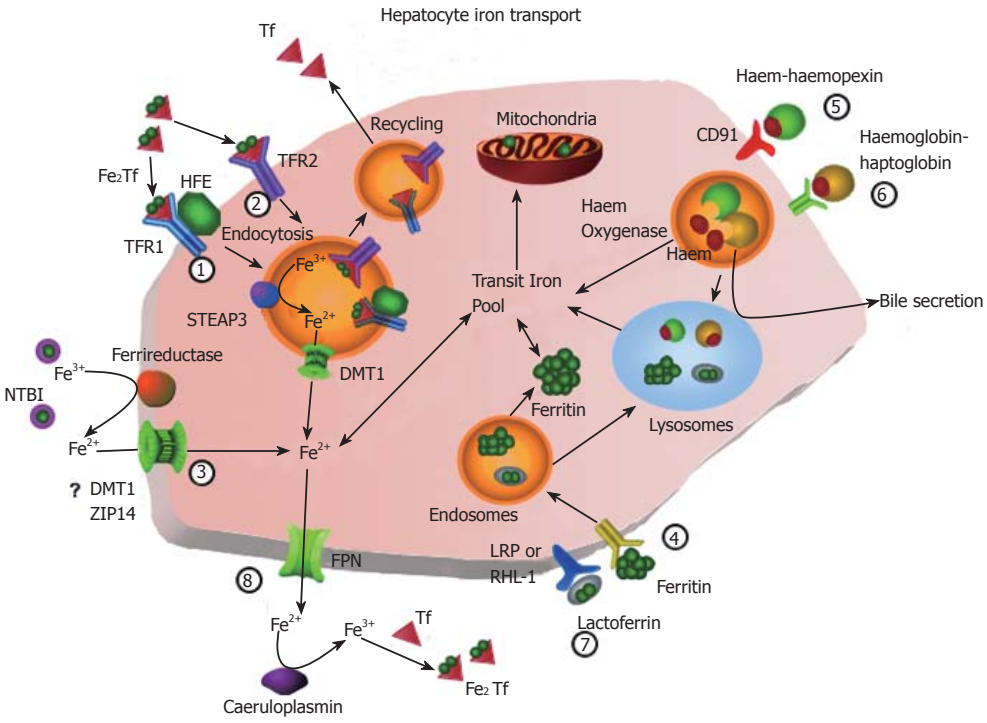

Figure 8: Iron transport in the liver (Graham, et al. 2007)

E. Treatment

Treatment of WD patients has two main aims: reduce the dietary intake and absorption of copper, and promote excretion of copper deposited in tissues. Several factors influence the course of the therapy, such as the stage at which the disease is diagnosed and the clinical manifestation observed (El-Youssef 2003). Several Cu chelating agents are currently being used such as Penicillamine, Zinc, and Ammonium tetrathiomolybdate. **Zinc** interferes with intestinal absorption of Cu and promotes metallothionein production, and it is recommended as first line therapy in neurological patients (Ferenci, et al. 2012). **Tetrathiomolybdate**, is limited to treating patients with early neurologic symptoms. It forms a complex with available copper and albumin to obstruct uptake of copper by cells. Although its side effects are milder than Penicillamine, with less frequent neurological deteriorations, **Trientine**, a polyamine, is less potent copper chelators than Penicillamine. It chelates copper by forming stable Trientine-Cu complexes in a planar ring (Dong and Wu 2012).

D-Penicillamine (PA) was first identified by Abraham et al in 1943 as a product of penicillin hydrolysis. Walshe et al introduced PA in the clinic as a copper-mobilizing agent for the treatment of patients with Wilson disease in 1956. PA is an amino acid with a thiol side chain that readily oxidizes to form various disulfides with proteins, cysteine, homocysteine, and PA itself (Perrett 1981). While PA exists as two enantiomers D and L, clinically only D isomer is used since L is toxic. It is marketed as Cuprimine® (250mg capsules) and Depen® (250mg tablets).

Although Penicillamine is the recommended treatment, it has various side effects

such as hypersensitivity, pyridoxine deficiency, suppression of bone marrow and autoimmune diseases. In some patients, PA has worsened their neurological symptoms (Scheinberg, et al. 1987).

In the presence of Cu, the thiol group of PA undergoes oxidation, producing H_2O_2 (Lipsky and Ziff 1980). In addition to treatment of WD patients, PA is used in patients with Rheumatoid Arthritis, cysteinuria, and heavy metal intoxication.

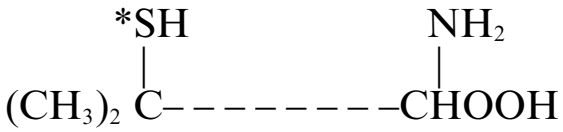

Figure 9: Structure of Penicillamine (*denotes thiol side chain).

Bathocuproine Disulfonate (BCS) is a non-toxic, non-permeable, copper specific chelator (Coloso, et al. 1990). BCS is used extensively in research to chelate copper in the media of cultured cells (Zischka, et al. 2011).

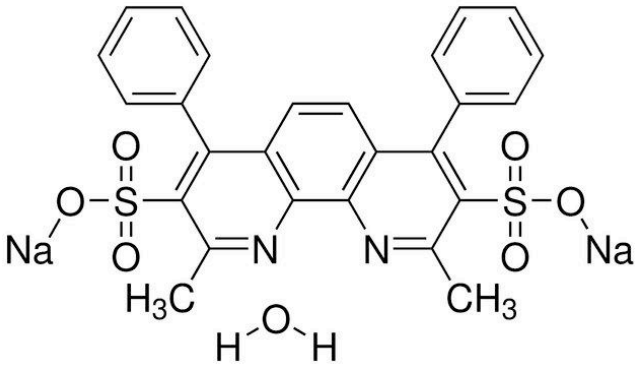

Figure 10: Structure of Bathocuproine Disulfonate

Analysis of sera from 5 WD patients who have been on PA treatment for more than 25 years showed extremely low levels of Cp protein in 4 of the patients and no detectable Cp oxidase activity (Macintyre, et al. 2004). The exaggerated hypoceruloplasminaemia of WD patients by long-term treatment with Cu chelating agents such as Penicillamine and Zinc, may result in iron overload (Medici, et al. 2007). Targeted disruption of murine Ceruloplasmin gene, showed progressive accumulation of iron and elevated serum ferritin, just like in patients with aceruloplasmineamia (Harris, et al. 1999). Moreover, 95% of mice fed a copper deficient diet, not only had low Cp activity and reduced hemoglobin, but also hepatic iron loading (Chung, et al. 2004). Few investigators demonstrated iron overload in the liver of compound heterozygous WD patients on PA treatment (Hayashi, et al. 2013; Schilsky 2001; Shiono, et al. 2001).

F. Objectives of the Study

WD patients have defective ATP7B gene which disturbs copper homeostasis and its loading into cupro-proteins leading thus to copper accumulation in liver. Ceruloplasmin (Cp) belongs to the multi copper oxidase family of proteins. It plays a key role in loading Fe into serum transferrin. Since copper mediates Cp ferroxidase activity, we postulate that in WD patients, lifelong treatment with copper chelators will decrease the level of Cu incorporation into apoceruloplasmin; thus affecting Cp ferroxidase activity and consequently disturbing iron homeostasis.

In this study we aim to investigate whether disturbances in Iron homeostasis are secondary to reduced Cp ferroxidase activity that may occur in WD patients treated with the copper chelator Penicillamine.

This will be addressed by examining:

- The *in vitro* effect of $CuSO_4$ and /or PA, treated HepG2 cells on

- Viability using MTT assay
- Toxicity using LDH cytotoxicity Kit, and ROS generation
- Mitochondrial Membrane potential and ETC enzyme activities
- Cell cycle analysis
- Oxidase activity of Cp using pPD spectrophotometric assay
- Expression of Ceruloplasmin, ferritin and transferrin levels

PA will be compared to BCS, a copper chelator not used as a drug.

- Fe level, Fe binding proteins and Cp ferroxidase in WD patients, treated with PA who are homozygous for mutation in the ATP7B gene. The following will be determined on recruited patients:

- Serum Fe, Cu levels
- Ferritin, Transferin levels
- Serum Cp ferroxidase activity using pPD assay and Cp ferroxidase kit.

CHAPTER II

MATERIALS AND METHODS

A. Materials

1. Cell Line

HepG2: Human liver cancer cells (cat# HB-8065), were originally purchased from the American Type Culture Collection, Manassas, VA, USA.

2. Disposable Lab Ware

- Cell culture plastic wares were purchased from corning

- Microscope cover glasses for immunofluorescent assays (cat # G15973C) were purchased from GAIGGER brand.

- Flow cytometry tubes, 5mL polysterene Round-Bottom Tube 12 x 75mm (cat# 352058) were purchased from BD Falcon.

3. Cell Culture Reagents

Cell culture reagents were purchased from Lonza: Roswell Park Memorial Institute (RPMI) (cat# BE12-115F); Dublecco's Modified Eagle's Medium (DMEM) (cat# BE12-741F); Fetal Bovine Serum (FBS) Heat Inactivated (cat# DE14-801F); Penicillin-Streptomycin mixture (PEN-Strep) (cat# P4333-110M0790); Trypsin (cat# BE 17-160E); Phosphate buffered saline (PBS) without calcium and magnesium (cat# BE17-517Q)

4. In Vitro Kits

Roche : Cell Proliferation Kit I (MTT) (cat # 11699709001); Cytotoxicity Detection Kit^{PLUS} (LDH) (cat# 04744934001)

ImmunoChemistry TECHNOLOGIES: Mito PT^{TM} Kit (JC-1) (cat# 911)

Uscn *Life Science Inc*: ELISA Kit For Ceruloplasmin (CP) (cat#SEA909Hu)

5. Chemicals and Reagents

Amersco: Polyxyethylene-20-Sorbitan Monlaurate (Tween; cat# 0777-1L); Sodium Dodecyl Sulfate (SDS); Dimethyl sulfoxide (DMSO)

BIO-Rad: N, N, N', N' tetra-methylethylenediamine (Temed); Glycine; acrylamide/bisacrylamide; Nitrocellulose membrane (2µm); Ammonium Per Sulfate (APS); and Sample Loading Buffer 5x; (Cat # 161-0767).

BDH laboratory reagent: Sucrose cat# 302997J

Fermentas*:* Page Ruler plus Prestained Protein Ladder cat # 0059013.

ICN: Hepes: (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) cat# 194550.

MERCK: Potassium Hydroxide pellets (KOH)

Roche: Enhanced ChemiLuminescence's Reagent (ECL) Kit

SIGMA: Tris-Acid; Tris-Base; Methanol; Triton 100x; ß-Mercaptoethanol; Isobutanol; Sodium pyruvate (cat # S8636); Ribonuclease A, (cat # R6513); Propidium Iodide, (cat# P4170); N- acetylcysteine (NAC); 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox); Nitro Blue Tetrazolium Chloride (NBT) (cat#N-6876); D-Penicillamine (cat#P-4875).

Scharlau: Ethylenediaminetetraacetic acid, EDTA, disodium salt, dihydrate (cat #AC0963)

FLUKA: Bathocuproinedisulfonic acid Disodium Salt (BCS) (cat #11870)

6. Antibodies

Abcam: Ceruloplasmin, rabbit polyclonal (ab48614); Ferritin, rabbit polyclonal (ab7332); Transferrin, rabbit polyclonal (ab1223).

Santa Cruz: GAPDH (6C5), mouse monoclonal antibody (Sc-32233)

7. Buffer Preparations

- Sucrose Hepes Tris–Base buffer , SHT, (250mM: 10mM: 50mM). Up to 1l with ddH_2O was prepared by dissolving 85.6g of sucrose, 2.83g of Hepes and 60g of Tris-Base and adjusting its pH to 7.4.
- Lysis buffer, SHT-Triton (0.001%): 1µl of triton (1%) added to 1mL of SHT.
- Laemmli (4X): 8mL of 10% SDS, 1mL of 100% glycerol, 100µl of 0.01% bromophenol blue and 2mL of ß-Mercaptoethanol.
- Acrylamide (30%): 29.2g acrylamide and 0.8g N, N-methylene-bisacrylamide were dissolved in 100mL ddH_2O and stored at 4^oC for a maximum of 2 weeks.
- Ammonium Per Sulfate (APS, 10%): 0.1g APS was dissolved in 1mL ddH_2O.
- Resolving gel (7%): was prepared by mixing 2.4 ddH_2O, 1.16 mL of 30% acrylamide, 1.3 mLTris- HCl (1.5M, pH 8.8), 50µl 10% SDS, 50µl APS (10%) and 2µl Temed.
- Resolving gel (15%): was prepared by mixing 1.1 mL H_2O, 2.5 mL of 30% acrylamide, 1.3 mL Tris- HCl (1.5M, pH 8.8), 50µl 10% SDS, 50µl APS (10%) and 2µl Temed.

- Stacking gel (4%): was prepared by mixing 1.4mL ddH_2O, 330µl (30%) acrylamide, 250µl Tris-HCL (0.5M, pH6.8), 20µl SDS (10%), 20µl APS (10%) and 2µl TEMED.
- Running buffer (5x, pH 8.3): was prepared by mixing 15.1 g of Tris-Base, 72 g of Glycine and 5 g of SDS in 1 liter ddH_2O.
- Transfer buffer (1X): 3.03 g of Tris-Base, 14.4 g of Glycine and 10 mL of 10%SDS were dissolved in 200mL methanol and completed to 1 liter ddH_2O.
- Blocking buffer (5%): 5g of fat free milk were mixed with 50 µl tween in 100 mL TBS.
- Tris-HCl (1M, pH 8): was prepared by dissolving 12.14g of Tris-HCl in up to 1 liter ddH_2O, adjusting the PH to 8.
- TBS (1X): 10mL Tris-HCl (1M, pH 8) and 30mL NaCl (5M) were mixed together with ddH_2O up to 1000mL.
- Washing buffer TBS-T: for each 100 mL TBS, 50µL Tween20 is added.
- Trichloroacetic acid (0.3%): 0.3g TCA was dissolved in 100mL ddH_2O.
- Stripping buffer: 6.25mL Tris-HCl (1 M), 20 mL SDS % were mixed and diluted up to100 mL ddH_2O.
- Potassium Hydroxide (KOH, 2M): 11.22 g of KOH were dissolved with ddH_2O up to 100 mL.
- Sodium Chloride (NaCl, 5M): 146.1g of sodium chloride were dissolved in 500mL ddH_2O under mild heating.

8. Equipments

The following equipments were used in the study:

- Centrifuge 5416 and centrifuge 5810 (eppendorf)
- Epson Expression 1680 Pro (Epson)
- Flow cytometer (Becton Dickinson, Research Triangle, NC)
- Fluroskan Ascent FL (Thermo).
- Fluorescent Microscope (OLYMPUS; BH2-RFCA)
- Hotplate stirrer (corning)
- Hematocytometer (Fisher scientific) (cat # 0267110)
- Inverted microscope (Axiovert 25)
- Lyophilizer (LABCONCO)
- Mini spin centrifuge (Thermo)
- Multiskan EX (Elisa reader) (Thermo).
- RP X-OMAT processor (Model M6B) (KODAK)
- AES-2010 Speed Vac System (Thermo Savant)
- UV-VIS scanning Spectrophotometer (UV-2101 PC) (SHIMADZU).

9. Human Subjects

Of the several Lebanese families that have been identified with members affected with WD, a total of 5 lebanese WD patients (P1,P2,P3,P4, and P5) from 2 unrelated families were recruited in our study. All the patients were diagnosed at AUBMC and were referred to Dr Barada. All had WD confirmed by clinical tests and genotypic analysis. Patients were eligible to participate in the study if they: 1- sign a consent form (protocol#BioCh.JU.01), 2- were homozygous for disease causing mutation, 3- had no mutation in Cp gene, 4- were not anemic and 5- are on copper chelators.

Subjects were then asked to have the following tests performed: CBC, serum Ceruloplasmin, ferritin, transferrin, and iron saturation levels, and serum copper and iron levels. Information about our subjects is summarized in table 3.

Subjects	Sex	Age	Age of diagnosis	Medication	Mutation in WD	Mutation in Cp
P1	female	12	12, by screening	Zinc	Ex 18: (AAT-AGT)-Asn1270Ser, homo	-
P2	female	16	10, by screening	Penicillamine	Ex 8: C ins pro767pro FS, homo	X
P3	female	20	5, by screening	Penicillamine	Ex 8: C ins pro767pro FS, homo	X
P4	male	33	16	Penicillamine	Ex 8: C ins pro767pro FS, homo	X
P5	male	40	5, by screening	Penicillamine	Ex 8: C ins pro767pro FS, homo	X

Table 3: WD patients recruited in the study

B. Methods:

To mimic the conditions of WD patients, cells were treated with Cu and/or copper chelators over a range of concentrations in order to determine the Cu concentration at which 50% of cell death occur and concentration of Cu chelators that shows no effect on viability.

1. Cell Culture

HepG2 cells were cultured at 37°C in DMEM (500 ml) supplemented with 10% FBS and 0.5% Pen-Strep using a humidified 5% CO_2 incubator. Cells were seeded in cell culture plates at a density of $1.2x10^5$ cells/ml for 24 hr, treated with each of $CuSO_4$, and/or PA/BCS at the appropriate concentration.

2. Viability Assay

HepG2 cells were seeded in 96 well plates at an average density of $1.2x10^4$ cells/100µl media. Effect of Cu, PA, and BCS on HepG2 cells were determined at final concentrations of (0-200 µM), 250 µM, and 250 µM respectively compared to a control. Viability was estimated using the MTT proliferation kit. Briefly, 10 µl of yellow tetrazolium salt was added to control and treated cells and incubated for 4 hr. Metabolically active cells reduce the salt into purple formazan crystals, which were solubilized by adding solubilizing reagent (100 µl) for an overnight. The intensity of the developed purple color was determined by measuring absorbance using ELISA reader at 595 nm. Viability was calculated as follows:

$$\% \text{ Viability} = \frac{\text{Abs (treated cells)} - \text{Abs (media + treatment)}}{\text{Abs (untreated cells)} - \text{Abs (media)}} \times 100$$

3. ***Intracellular ROS level determination***

To determine whether ROS mediates cell death observed in treated HepG2 cells , the level of ROS generated was determined using P-Nitro-Blue-Tetrazolium Salt (NBT) assay. HepG2 cells ($1.2x10^4$ cells/100 µl) were seeded in 96 well plate and treated for 24 hr. Media was then aspirated and 100ul of NBT (1mg/ml) was added for 1 hr at 37°C. Cells were then washed with methanol and left to dry. The reduced NBT by ROS, yields formazan crystals that were solubilized by adding KOH (2M, 120 µl) then DMSO (140 µl). ROS producing cells display faint turquoise color as contrasted by the control that produces an intense turquoise color. Absorbance was read at 630nm using ELISA reader. ROS level was calculated as follows:

$$\% \text{ NBT Reduction} = \frac{\text{Abs (treated cells)}}{\text{Abs (control)}} \times 100$$

$$\% \text{ ROS production} = 100 - \%\text{NBT reduction}$$

4. ***Cytotoxicity Assay***

To examine if Cu and/or copper chelator treatment disturb the plasma membrane integrity, cytotoxicity of various treatments was assessed in treated vs control HepG2 cells, by measuring LDH release, using the Cytotoxicity Detection kit ^{plus} (LDH). HepG2 cells ($1.2x10^4$ cells /100 µl) were seeded in 96 well plate. Two sets of triplicates were used for control: "low control" (LC) with minimal LDH release, and "high control" (HC) with

maximal LDH release induced by 5 µl of lysis reagent provided by the kit. Then 100 µl /well of reaction mixture was added on LC, HC, and treated cells, and incubated for 20 min. To terminate the reaction, 50 µl of stop solution was added and the orange-red color read at 492 nm on ELISA reader. LDH release was calculated as follows:

$$\text{Cytotoxicity (\%)} = \frac{\text{Abs (treated cells)} - \text{Abs (LC)}}{\text{Abs (HC)} - \text{Abs (LC)}} \times 100$$

5. ***Immunofluorescent detection of Mitochondrial Depolarization (Mito JC-1)***

Mitochondrial changes including derangement of function were reported in WD patients, we examined the effect of Cu and/or Cu chelators on mitochondrial membrane potential. Loss of electrochemical gradient across the mitochondrial membrane may be assessed using the cell permeable lipophilic fluorescent dye known as JC-1. (5, 5', 6, 6' – tetrachloro – 1, 1', 3, 3' – tetraethyl – benzamidazolocarbocyanin iodide). The delocalized positive charge on JC-1 favors the entry of the dye into the negatively charged non-apoptotic mitochondria, forming orange fluorescent aggregates. However, in compromised cells, JC-1 disperses throughout the cell forming green fluorescent monomers. HepG2 cells were seeded on sterile glass cover slips in 12 well plates (1.2×10^5/ml media). Following media aspiration, the stain (300 µl /well) was added to the cells and incubated at 37°C for 15 min, after which cells were washed twice (10 min each) with washing buffer. Finally, the cover slips were mounted on microscope slides, left to dry, and visualized using fluorescent microscope (OLYMPUS; BH2-RFCA); images were taken using OLYMPUS MICRO software.

6. *ETC Enzyme Activity*

Derrangement of mitochondrial function and dissipation of membrane potential may be a consequence of ETC enzyme inhibition. The effect of Cu and/or Cu chelators on ETC enzyme complexes was tested.

- *Complex I (NADH Dehydrogenase) Activity:*

The effect of $CuSO_4$ and/or PA/BCS on activity of NADH dehydrogenase in HepG2 cells was determined. Briefly, in a final volume of 800 µl of TPE buffer, cell lysate equivalent to 0.2 mg was added. The reaction was initiated by adding 5 µl NADH (30 mM). The variation in absorbance was monitored for ten minutes at 340 nm. Residual enzyme activity in treated cells was expressed as ΔAbs/mg/min compared to a control considered as 100% activity.

- *Complex II (Succinate Dehydrogenase) Activity:*

Complex II activity was assayed spectrophotometrically by monitoring the change in absorbance of $K_3Fe(CN)_6$ reduction with time. Cell lysate protein (0.2 mg) of treated and untreated HepG2 cells were introduced into a microcuvette containing in a final volume of 800 µl: KCN (9mM, 75 µl), TrisHCl (90 mM, pH 7.2, 75 µl), sodium succinate (93 mM, 50 µl), and dd.H_2O (475 µl). The reaction was initiated by adding $K_3Fe(CN)_6$ (10 mM, 75 µl). The variation was recorded for 30 min at 400 nm, and enzyme activity was expressed as ΔAbs/mg/min, compared to a control considered as 100% activity.

7. *Cell Cycle Analysis*

The effect of Cu and Cu chelator on cell cycle phases was examined. Propidium Iodide (PI) stains DNA and its fluorescence intensity is directly proportional to DNA content in a cell. Proportion of cells in the different cell cycle phases is thus assessed by DNA fluorescence using a flow cytometer.

Cells (1.2 million/ 9mL) were seeded in 100mm Petri dishes, treated with Cu and Cu/PA, and examined after 24 hr. As follows, cells were scraped, collected, and centrifuged (1500 rpm, 5 min). The pellet was washed 2x with ice- cold PBS (1X, 1mL), fixed by the consecutive resuspension in ice-cold PBS (1x, 1mL) and absolute ethanol (3mL, -20°C). Fixed cells were stored at –20°C before staining (they are stable up to 10 days before staining).

Fixed cells were warmed to room temperature, and centrifuged (1500 rpm, 5 min). Pellet was washed once with ice-cold PBS (1X, 1mL), resuspended in RNase A (200 µg/mL, 100 µl), incubated (1 hr) in water bath (37°C), and centrifuged (2000 rpm, 5 min). The obtained pellet was resuspended in PBS (500 µl) and transferred into polystyrene round bottom Falcon tubes (5 mL). Propidium iodide stain (1 mg/mL, 30 µl) was added to the cells, vortexed, incubated (10 min) in the dark, and stored at 4°C until read by flow cytometer after 2-3 days.

Cell cycle analysis was performed using Fluorescence Activated Cell Sorter (FACS scan) flow cytometer (Becton Dickinson, Research Triangle, NC). Each sample was collected as 10,000 ungated events and the corresponding cell cycle distribution, according to the DNA content, was determined using Cell Quest software (Becton- Dickinson).

8. ***Western Blot Analysis***

Effect of Cu and/or Cu chelators on the expression of ceruloplasmin, ferritin, and transferrin was examined.

- *Cell treatment and cell lysate preparation*

HepG2 cells (1.2 million / 10 ml) were seeded in petri dishes and treated with $CuSO_4$ (50 μM and 100 μM); BCS (250 μM); PA (250 μM); $CuSO_4$/BCS (50/ 250 μM); $CuSO_4$ /PA (50 /250 μM) for 24 hr. Cells were scraped on ice and centrifuged (1200 rpm, 7 min). The pellet was washed twice with ice-cold PBS, resuspended in SHT-Triton lysis buffer, and triturated. Protein quantification was done following the Bradford assay. Samples were mixed with 4x Laemmli, and denatured by boiling for 10 minutes.

- *Western Blot*

Samples (100 μg protein) were loaded on SDS-PAGE and allowed to migrate in 1x running buffer. Separation of the proteins Ceruloplasmin, Transferrin, and Ferritin was performed on 7%, 10%, and15% gels, following which proteins were transferred using BIORAD electro-transfer set up onto PVDF, PVDF, and nitrocellulose membranes respectively. Membranes were blocked with 10% milk for 1 hr, and overnight incubation with the primary antibody in cold room diluted at: (Ceruloplasmin [1:300]; Ferritin [4:3000]; Transferrin [1:1000]). Membranes were then washed with TBS-Tween three times (10 min each) and then incubated with the appropriate secondary antibody (Anti-mouse for GAPDH [1:10,000], and anti-rabbit for Ceruloplasmin, Ferritin, and Transferrin [1:10,000]).

Protein expression of treated and control cells were visualized using enhanced chemiluminescence (ECL kit), and protein bands were normalized to GAPDH using ImageJ software and expressed as percent expression.

9. *Ceruloplasmin Ferroxidase Activity*

Fe homeostasis involves ceruloplasmin ferroxidase activity that is mediated by Cu loading into apoCp. The holoCp would catalyze then oxidation of Fe^{+2} into Fe^{+3} favoring its loading into transferrin. We examined in this part the effect of Cu and/or Cu chelators on ferroxidase activity. The serum of WD patients, as well as HepG2 treated cells were assayed using two techniques:

- *Ceruloplasmin (Ferroxidase) Kit (ELISA Kit)*

Following centrifugation and separation of plasma, protein quantification was performed and samples were diluted 5000 fold in PBS. All reagents, samples, and standards were prepared as described per the instruction manual of the Ceruloplasmin (CP) Ferroxidase kit. The assay is based on the principal of the formation of an oxidized product by Ceruloplasmin (found in serum) with a substrate (provided by the kit) that yields a product that can be read at 450 nm.

The absorbance read by the WD patients was compared to the control after standardizing with the amount of protein in each sample.

- *Ceruloplasmin pPD Oxidase Assay*

Ceruloplasmin ferroxidase activity was determined following the spectrophotometric pPD oxidase assay described (Sunderman and Nomoto, 1970).

Ceruloplasmin catalyzes the oxidation of pPD (P-Phenylenediamine) to produce a colored product that can be spectrophotometrically detected at 530 nm. A 100 µg protein sample (plasma and cell lysate 2 – 10 µl) were added to a final volume of 1 ml Acetate buffer (0.1M, pH 5.45). Oxidase reaction was initiated by adding freshly prepared pPD (500 µl, 0.1%) and incubating in the dark at 37°C. The reaction was stopped 30 minutes later by adding 30 µl of sodium azide (1.5 M). Absorbance was read against a control of acetate buffer.

10. ***Statistical Analysis***

SPSS software was used to analyze data and determine significance. Both One-Way Anova and Independent sample t-test were applied. For One-Way Anova analysis, Post Hoc tests, Bonferroni and Scheffe were used for multiple comparisons. p-value < 0.05 is considered significant. For each parameter tested, a set of at least three experiments were done where a triplicate of three determinants was applied in each experiment. Moreover, for each parameter, both, inter-categorical statistical significance and significance relative to control were analyzed.

CHAPTER III

RESULTS

A. Effect of $CuSO_4$ on HepG2 viability

$CuSO_4$ exerted a significant ($p<0.001$) dose-dependent decrease in viability of HepG2 (Figure 1a). Treatment of cells for 24 hr with $CuSO_4$ (10 μM - 200 μM) induced significant cell death reaching a maximum of 100% at 200 μM ($p<0.001$). A 50% cell death was obtained at 50 μM $CuSO_4$ ($p<0.001$).

Further treatment of HepG2 with $CuSO_4$ at EC50 for different times (0.5 – 24 hr) showed no significant difference (Figure 1b).

B. Effect of Copper chelators on viability

The protective effect of copper chelators PA and BCS on Cu-treated HepG2 cells was investigated (Figure 2). Co-treatment of HepG2 cells with Cu (EC50) and PA (250 μM) exhibited no protective effect, instead it further decreased the viability by 70% ($p<0.001$). However, co-treatment of HepG2 cells with Cu (EC50) and BCS (250 μM) was protective restoring the viability to 100%. Treatment of HepG2 cells with either PA or BCS did not influence the viability.

Concentrations below 250 μM were used for both PA and BCS with no significant results (data not shown).

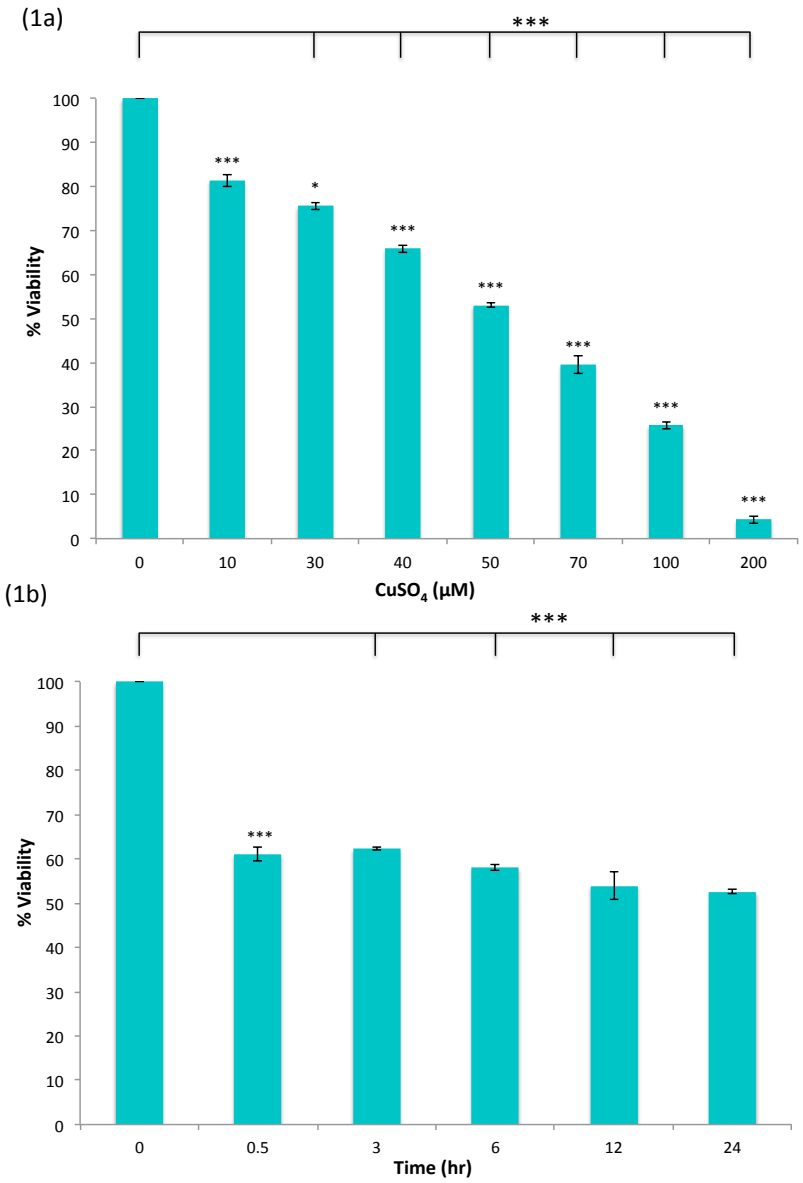

Figure 1: (a) Effect of varying $CuSO_4$ concentrations on viability in HepG2 cells. (b) Time effect of $CuSO_4$ on viability. Cells were treated with 50 µM $CuSO_4$ and left for different incubation periods. Data presented is the mean ± SEM of 9 determinations from three different experiments. Asterisks on bars represent inter-categorical statistical significance (each category with the preceding one), and those drawn up wards represent significance relative to the control. (*), (**), and (***) correspond to $P<0.05$, 0.01, and 0.001 respectively.

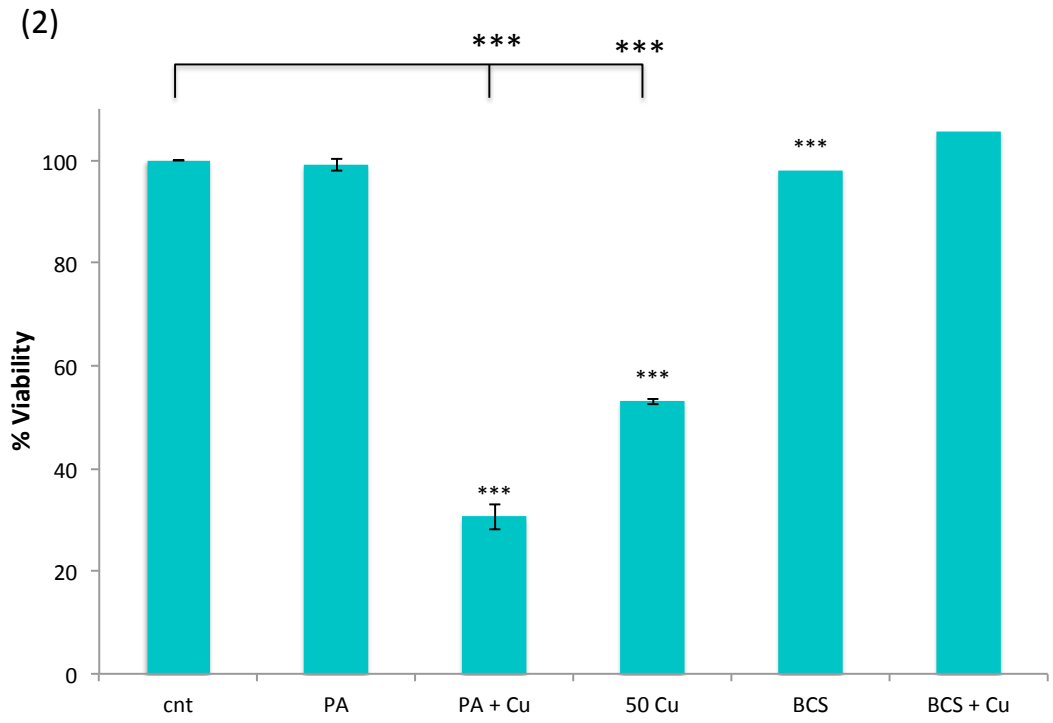

Figure 2: Effect of copper Chelators on HepG2 viability. Cells were co-treated with 50 µM $CuSO_4$ and 250 µM of either PA or BCS for 24 hr. Data presented is the mean ± SEM of 12 determinations from four different experiments. Asterisks on bars represent inter-categorical statistical significance (each category with the preceding one), and those drawn up wards represent significance relative to the control. (*), (**), and (***) correspond to $P<0.05$, 0.01, and 0.001 respectively.

C. Effect of Cu and Cu chelators on ROS generation

The level of ROS was determined in HepG2 cells treated with Cu (EC50), PA/BCS, or co-treated with Cu and either PA or BCS (Figure 3). Treatment of HepG2 cells with PA showed no effect, however, co-treatment of HepG2 cells with Cu (EC50) and PA (250 μM) reduced NBT by 44% ($p<0.001$), indicating increase in ROS level (56%). On the other hand BCS alone or co-treated with Cu exerted no effect on ROS generation. No ROS was produced upon treatment with various concentrations of Cu (24, and 48 hr).

D. Effect of Cu and Cu chelators on morphology of HepG2 cells and membrane integrity

The morphology of HepG2 cells treated with Cu, PA, BCS, and co-treated with Cu-PA/BCS was investigated (Figure 4). None of the treatments (Cu, PA, BCS, and Cu-BCS) had an effect on the morphology of HepG2 cells except for the co-treatment with Cu and PA where HepG2 cells lost their distinct shape, were rounded, and less in number.

Further investigation on cytotoxicity of Cu and Cu-PA/BCS on HepG2 cells was conducted. LDH release is a measure of cytotoxicity and cell lysis. There was no significant LDH release in HepG2 cells treated with Cu, PA, BCS, or in cells co-treated with Cu and either PA or BCS (Data not shown).

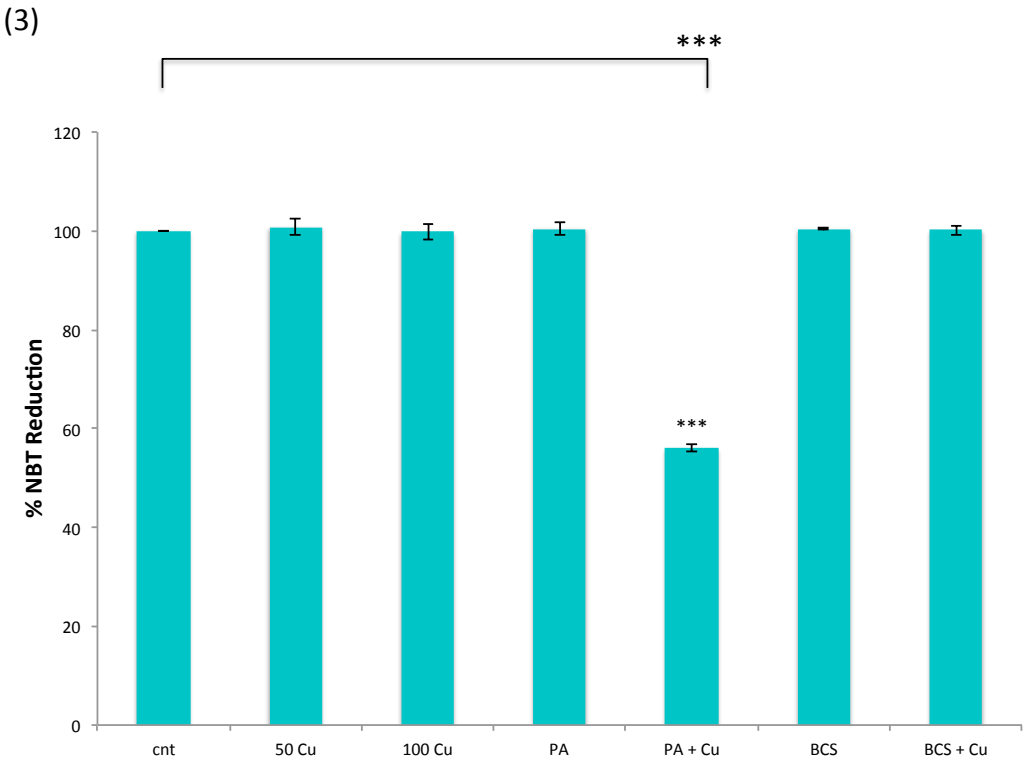

Figure 3: Effect of Cu and Cu chelators on ROS production. Cells were co-treated with 50 µM $CuSO_4$ and 250 µM of either PA or BCS for 24 hr. Data presented is the mean ± SEM of 9 determinations from three different experiments. Asterisks on bars represent inter-categorical statistical significance (each category with the preceding one), and those drawn up wards represent significance relative to the control. (*), (**), and (***) correspond to P<0.05, 0.01, and 0.001 respectively.

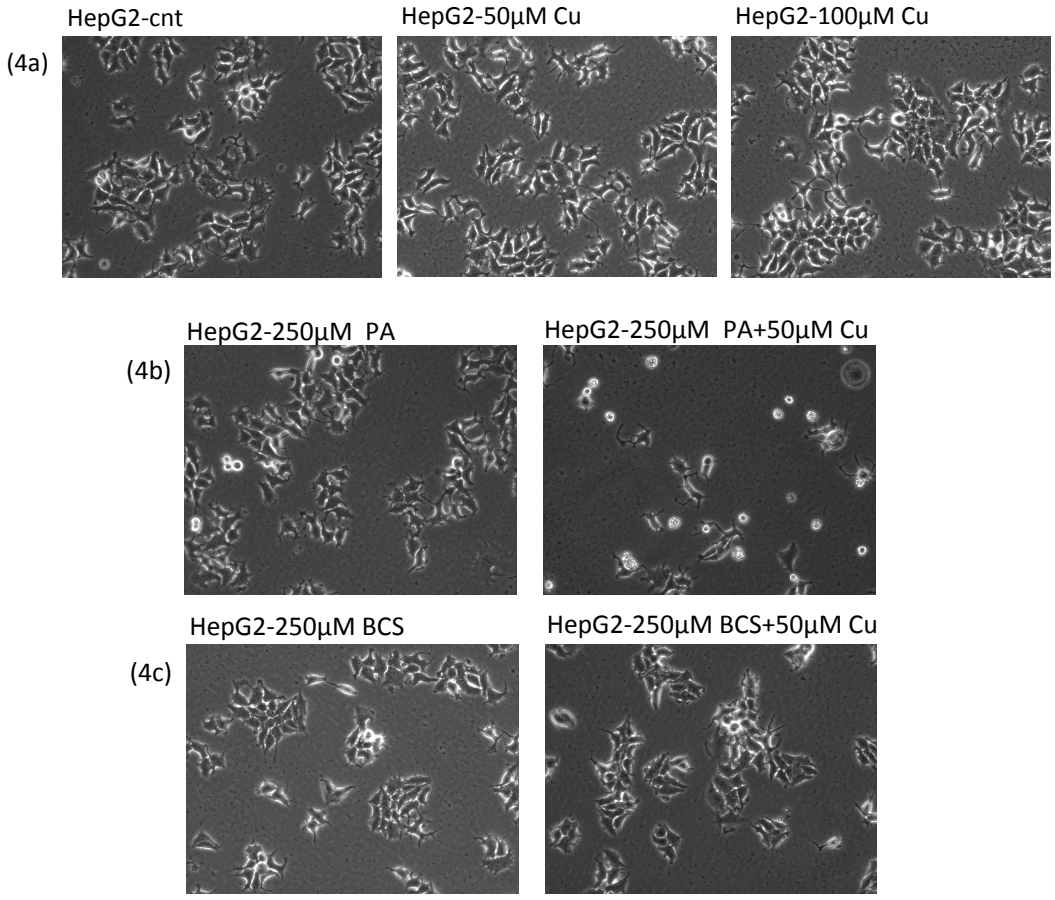

Figure 4: Effect of (a) $CuSO_4$, (b) PA and PA+ Cu, and (c) BCS and BCS+ Cu on morphology of HepG2 cells. HepG2 cells were seeded in 6 well plates and treated for 24 hr.

E. Cu-PA induces cell cycle arrest at the G2M phase

Cell cycle distribution of Cu and Cu-PA treated HepG2 cells was investigated (Figure 5). The average distribution of Cu treated HepG2 cells in the cell cycle phases G0, G1, S, and G2M was observed to be similar to HepG2 cells treated with Cu (50 µM, 100 µM). However, a sharp increase (63%) in the G2M phase was observed in HepG2 cells co-treated with Cu-PA.

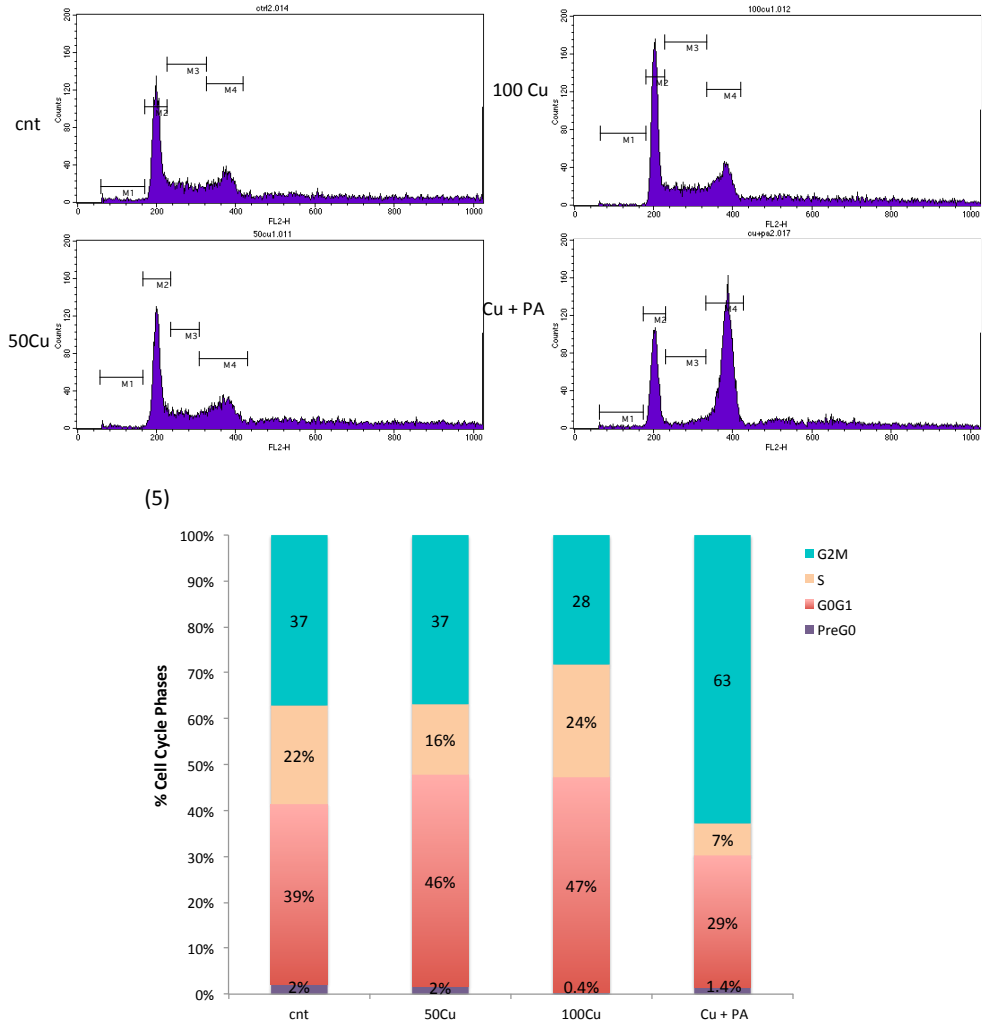

Figure 5: Cell Cycle analysis of HepG2 cells treated with Cu and Cu-PA. Cell cycle analysis was performed using Fluorescence Activated Cell Sorter (FACS scan) flow cytometer (Becton Dickinson, Research Triangle, NC). (Average of 2 experiments)

F. Depolarization of Mitochondrial membrane by Cu and/or Cu chelators

The effect of Cu (50 μM, 100 μM), PA (250 μM), BCS (250 μM), and Cu-PA/BCS on mitochondrial membrane potential was examined using MitoPT-JC assay (Figure 6). HepG2 cells treated with Cu showed a dose dependent polarization of mitochondrial membrane. Partial depolarization was observed with HepG2 cells treated with PA alone, whereas, complete depolarization was obtained upon co-treatment with Cu-PA, with a distinct change in the morphology and number of cells (Figure 6a). Treatment with BCS alone induced partial depolarization that was also seen in cells co-treated with Cu-BCS (Figure 6c).

G. Effect of Cu and Cu chelators on ETC enzyme activity

Residual activity of ETC enzymes Complex I and II was determined in HepG2 cells treated with Cu, PA, BCS, and co-treated with Cu-PA/BCS (Figure 7)

Treatment of HepG2 cells with Cu (50 μM) and (100 μM) decreased the activity of Complex I by 6% and 21% ($p<0.001$) respectively. Cells treated with PA or Cu-PA lowered the activity by 12% ($p<0.001$). BCS decreased the activity of Complex I by 31%, which was partially restored by co-administration with Cu to 10% (Figure 7a).

On the other hand, treatment of HepG2 cells with Cu (50 μM) and (100 μM) decreased activity of complex II by 27% ($p<0.001$) and 22% respectively. Each of the Cu chelators PA and BCS reduced complex II activity by 20% and 32% ($p<0.001$) respectively. While co-treatment with Cu-PA and Cu-BCS reduced the residual activity of Complex II by 50% and 30% respectively (Figure 7b).

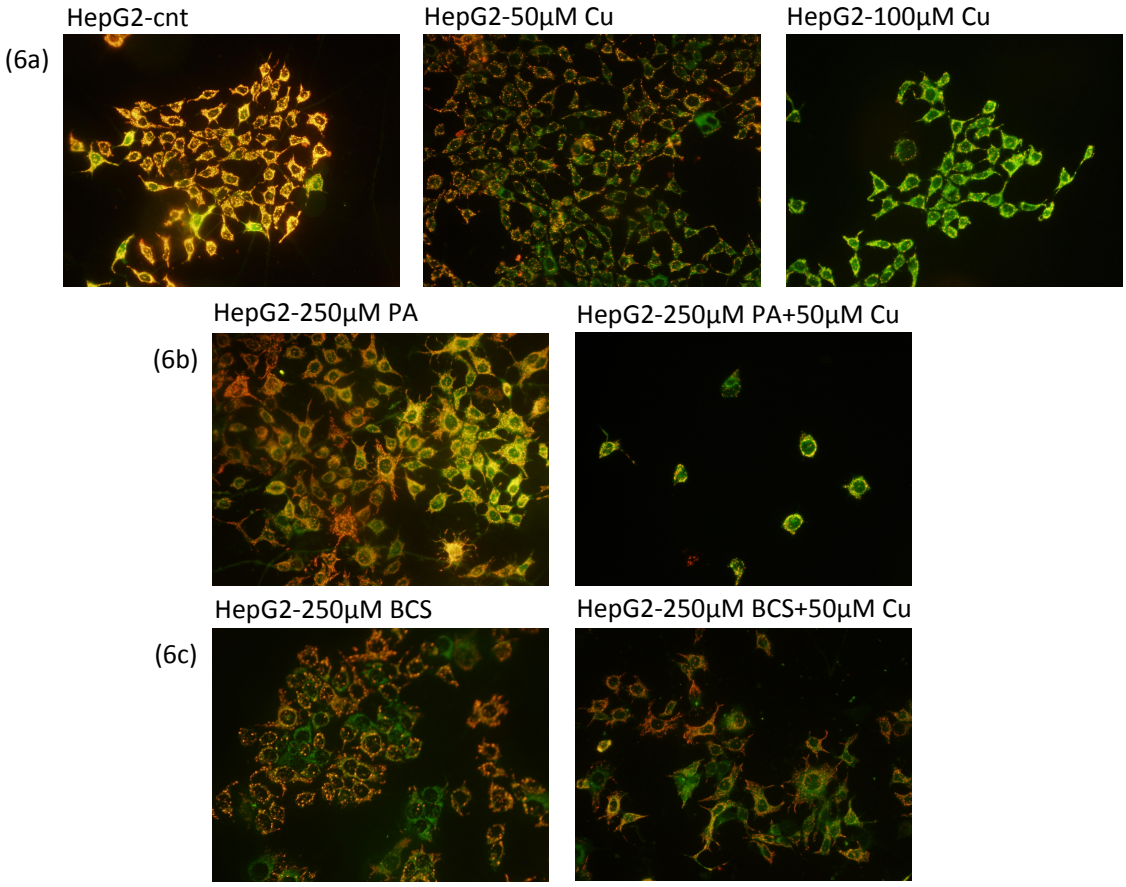

Figure 6: Effect of $CuSO_4$, PA, and BCS on mitochondrial membrane potential of HepG2 cells. Mitochondrial depolarization was detected by MitoPT JC-1 dye that fluoresces orange in healthy mitochondria and green in compromised ones. Representative figure of cells treated with: (a) $CuSO_4$ (b) PA and PA+ Cu, and (c) BCS and BCS+ Cu.

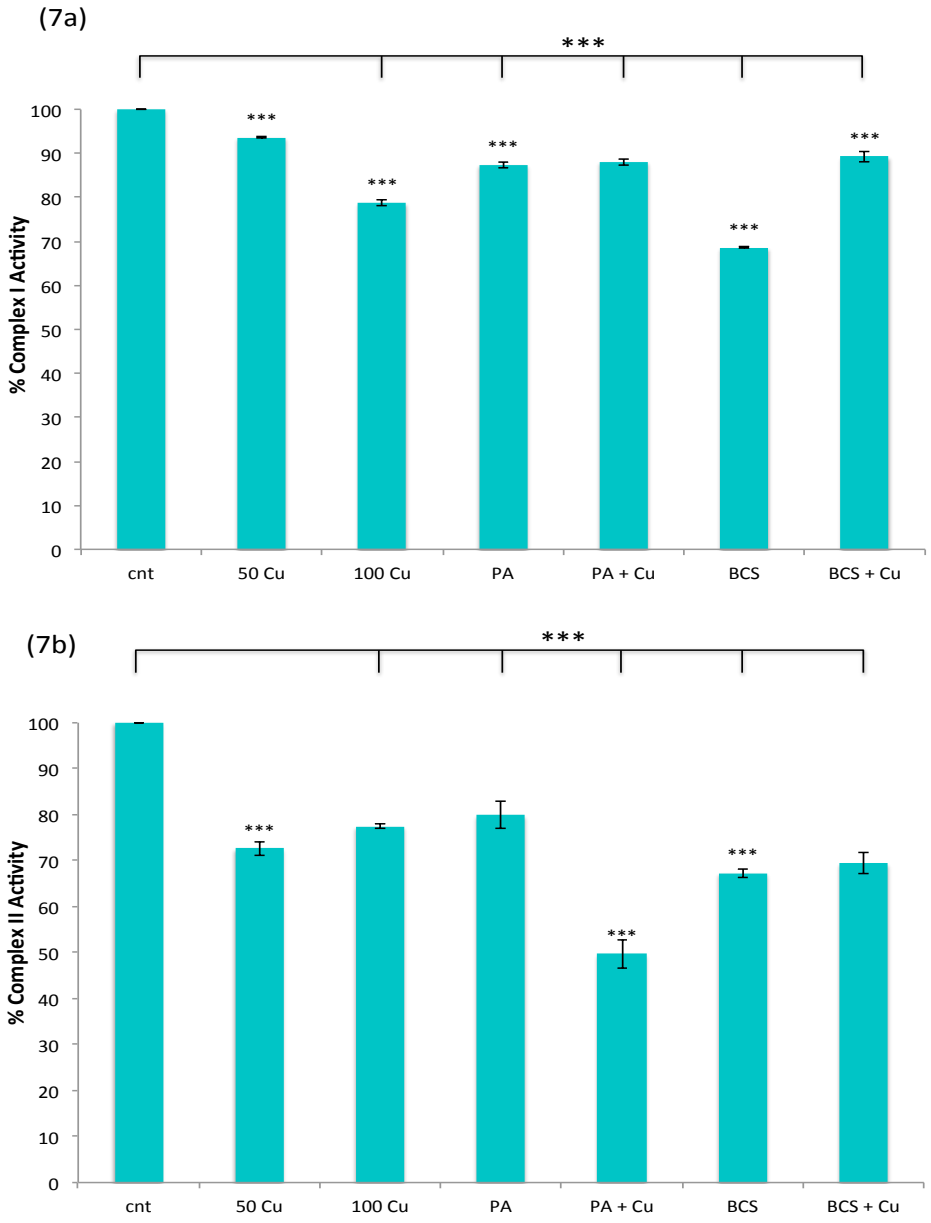

Figure 7: Effect of Cu and Cu Chelators on (a) Complex I and (b) Complex II residual activity. Data presented is the mean ± SEM of 3 determinations from three different experiments. Asterisks on bars represent inter-categorical statistical significance (each category with the preceding one), and those drawn up wards represent significance relative to the control. (*), (**), and (***) correspond to $P<0.05$, 0.01, and 0.001 respectively.

H. Effect of Cu and Cu chelators on expression of Ferritin, Ceruloplasmin, and Transferrin

The effect of Cu and Cu chelators on expression of iron and copper binding proteins (ferritin,Cp, Tf) was determined by using Western Blotting (Figure 8).

Ferritin expression was decreased by 8% and 14% ($p<0.05$) in Cu (EC50) and Cu/PA treated HepG2 cells respectively, and increased by 12% ($p<0.05$) in cells treated with BCS (Figure 8a).

On the other hand, Ceruloplasmin expression was increased upon treatment with Cu (50 μM) and (100 μM) by 9% and 12% ($p<0.01$) respectively. PA significantly decreased expression of Cp in HepG2 cells by 30% ($p<0.001$), while BCS by 9% ($p<0.05$) (Figure 8b).

Transferrin expression was also examined; our preliminary qualitative results show decrease in Tf expression in PA and Cu/PA treated cells. Inability to quantitatively assess the decrease is due to the background around the Tf bands due to non-specificity of the Tf polyclonal antibody (Figure 8c).

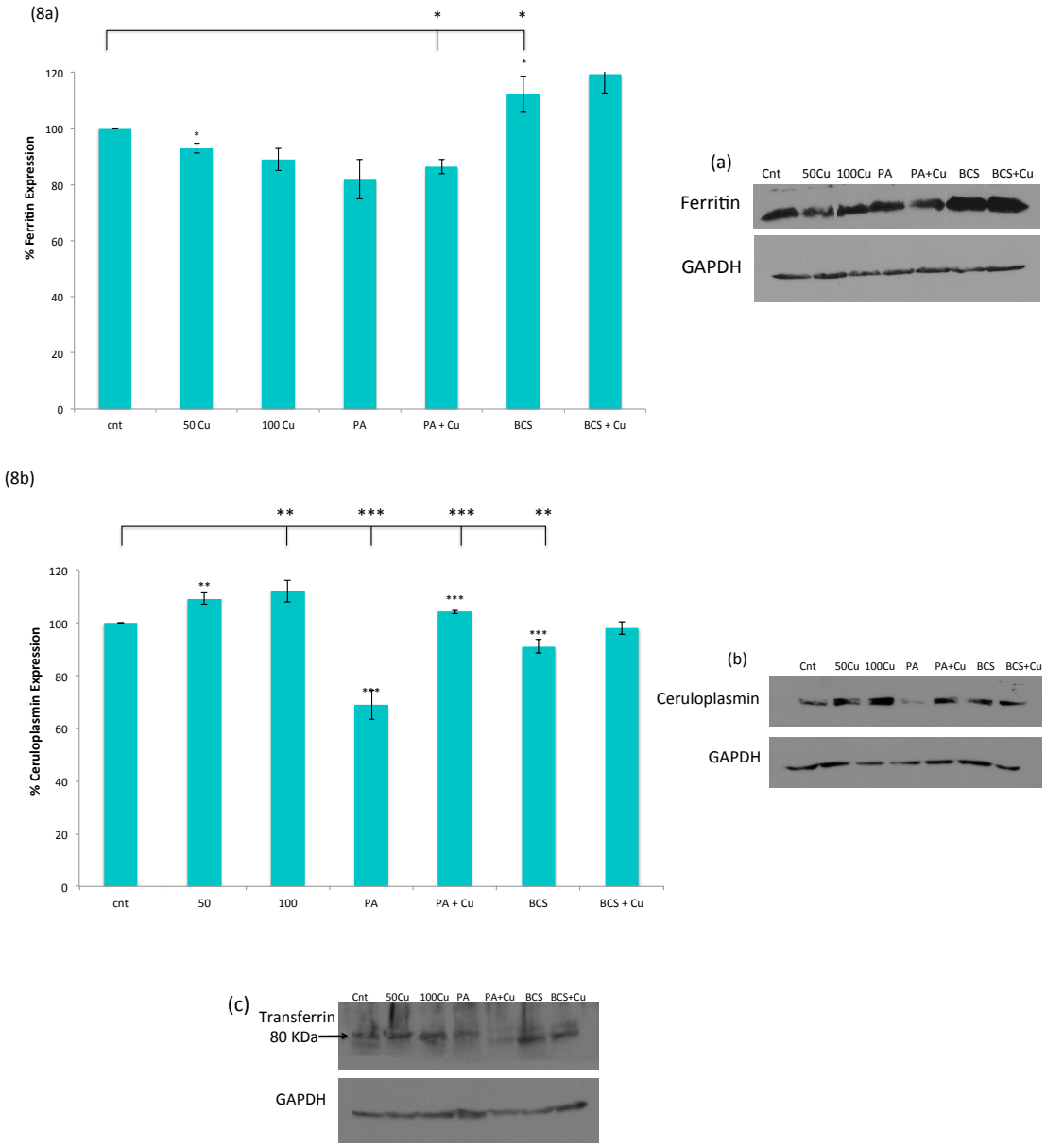

Figure 8: Effect of $CuSO_4$, PA, and BCS on expression level of (a) Ferritin (b) Cp and (c) Tf. Protein expression was assayed using western blot analysis. Quantitative assessment of bands was done using ImageJ. Expression levels of treated samples and controls were normalized to their respective GAPDH, then fold expression was determined relative to the control. All bars represent mean ± SEM. Asterisks on bars represent inter-categorical statistical significance (each category with the preceding one), and those drawn up wards represent significance relative to the control. (*), (**) and (***) correspond to $P<0.05$, 0.01, and 0.001 respectively. Transferrin is representative of 2 experiments.

I. Effect of Cu chelators on the pPD Oxidase activity of Ceruloplasmin in HepG2 cells

P-Phenylenediamine (pPD) Oxidase activity, was performed on HepG2 cells treated with Cu, PA, BCS, and co-treated with Cu-PA/BCS (Figure 9). HepG2 cells treated with Cu (EC50) showed no change in Ferroxidase activity compared to control HepG2 cells. PA and BCS treatment reduced the activity by 11% and 22% respectively ($p<0.001$). In Cu-PA and Cu-BCS treated cells, the oxidase activity decreased by 12% and 10% respectively ($p < 0.001$).

(9)

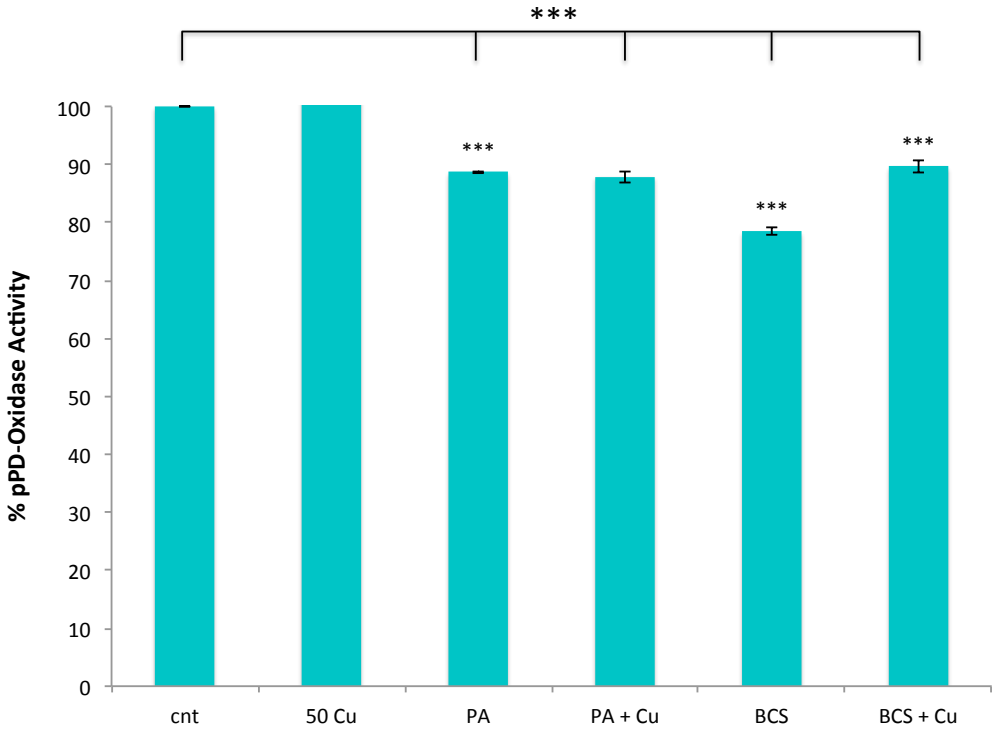

Figure 9: Effect of $CuSO_4$, PA, and BCS on pPD oxidase/Ferroxidase activity of HepG2 cells. Absorbance read at 530 nm of treated cells was normalized with control. Data presented is the mean ± SEM of 9 determinations from three different experiments. Asterisks on bars represent inter-categorical statistical significance (each category with the preceding one), and those drawn up wards represent significance relative to the control. (*), (**), and (***) correspond to $P<0.05$, 0.01, and 0.001 respectively.

J. Iron binding proteins and ferroxidase activity in WD patients

1. Ceruloplasmin, Ferritin, Transferrin, Cu and Fe levels in WD patients

Subjects	Fe ug/dl	Cu ug/dl	Fe binding capacity ug/dl	% Fe saturation	Transferrin g/l	Ferritin ng/ml
	37-160	70-155	270-450	15-50	2.00-3.60	Male: 25-310 Female Premeno: 12-150
P1	47	8	349	13.5	2.74	54.8
P2	73	29	386	18.9	3.03	235
P3	77	22	284	27.1	2.10	380
P4	92	<0.64	235	39	1.83	108
P5	104	26	294	35	2.42	291

Table 4: Clinical data of WD patients

Clinical data on Iron level and Iron saturation profile presented in the table on recruited patients, showed no anemia, a mild decrease in Fe binding capacity in (P3, P4, P5), a low transferrin level in P4 and mild elevation in Ferrin in (P2, P3) subjects.

2. Ferroxidase Activity

- *using pPD assay*

pPD oxidase assay was applied on the serum of 5 homozygous and 1 heterozygous WD patients and 4 controls (Figure 10a). Oxidase activity in normal controls ranged between (0.022-0.027 units), whereas in patients homozygous for the disease causing mutation, it ranged between (0.007-0.01 units). In one heterozygous patient, oxidase activity was 0.018 units. A reduction of 70% ($p<0.001$) in oxidase activity was obtained

comparing the average of control to patients (refer to Figure 10a-insert) (unit = Absorbance/µg of protein).

- *using Ferroxidase assay kit*

Ceruloplasmin Ferroxidase activity (Elisa kit) was used on the serum of 4 homozygous WD patients (Figure 10b). Ferroxidase activity in normal controls ranged between (0.007-0.009 units), whereas in the patients it ranged between (0.003-0.005 units)

Comparing the averages of controls to patients showed 50% ($p < 0.001$) decrease in ferroxidase activity in WD patients.

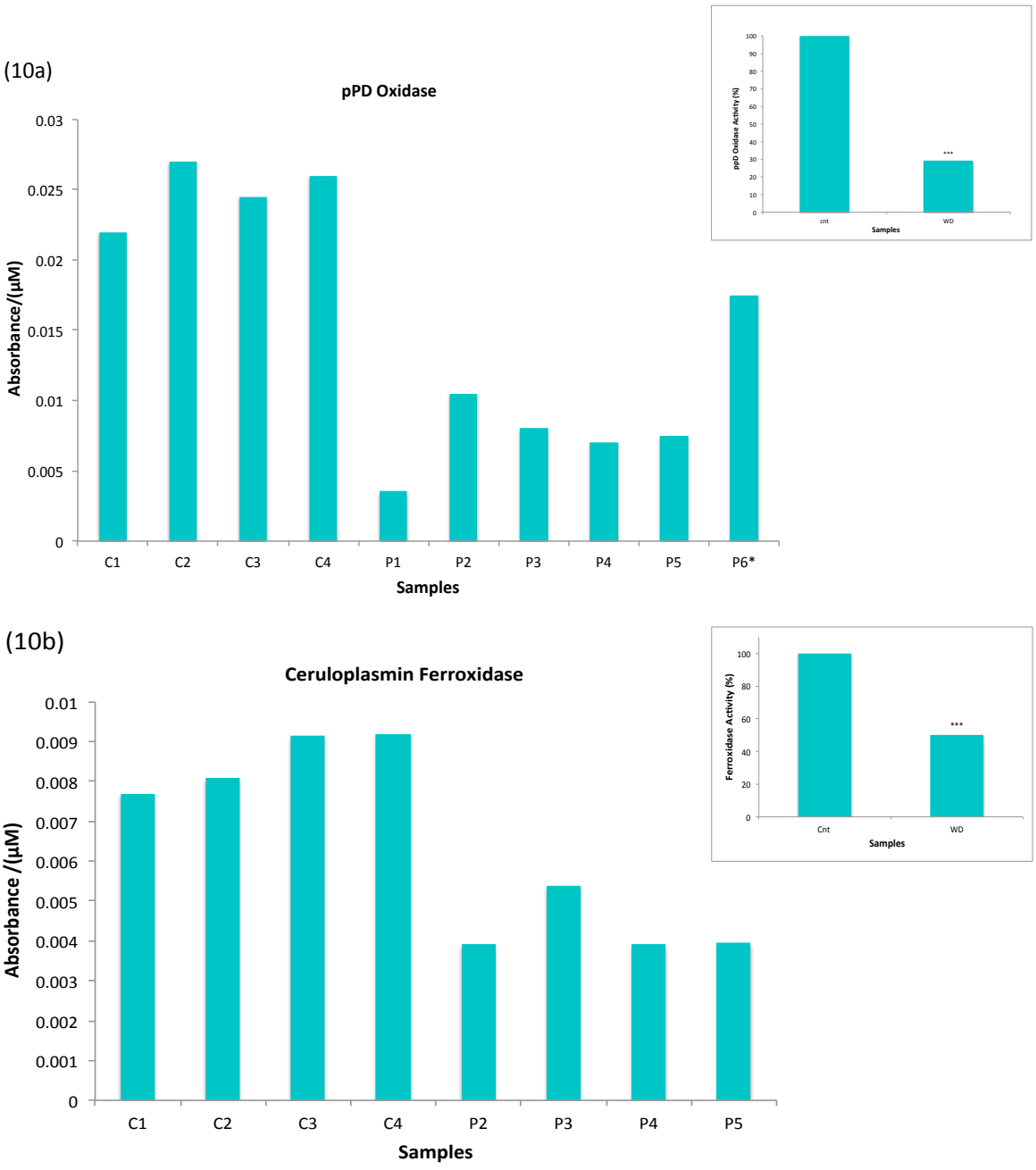

Figure 10: Ferroxidase activity of Wilson Disease patients was assayed using (a) pPD Oxidase activity and (b) Ceruloplasmin Ferroxidase activity kit. . Data presented is the mean ± SEM of 4 determinations. Asterisks on bars represent inter-categorical statistical significance. (*), (**), and (***) correspond to $P < 0.05$, 0.01, and 0.001 respectively.

CHAPTER IV

DISCUSSION

Copper is an essential micronutrient with structural and catalytic roles in various enzymes, yet toxic if present in excess. In this study, we investigated the effect of Cu and a copper chelator, Penicillamine (PA) on HepG2 viability and the expression of iron binding proteins ferritin, transferrin, and ceruloplasmin (Cp). We also compared PA to Bathocuproine Disulfonate (BCS), a known Cu chelator that is not used as a drug. In addition, the effects on Cp oxidase activity were examined with possible implications on Wilson Disease (WD) patients.

In WD patients, biliary excretion is impaired resulting in deposition of Cu in the liver. Hence, patients are recommended a lifetime treatment with Cu chelators such as Penicillamine, Trientine, and Zinc. However, this may limit further availability of copper resulting in suppressed incorporation of Cu into cuproproteins. Cu in Cp is indispensable for Cp ferroxidase activity that oxidizes Fe^{+2} into Fe^{+3} prior to its incorporation into serum transferrin. Thus, reduction in ferroxidase activity may lead to iron overload in WD patients. In this study, the *in vitro* effect of Cu/PA on HepG2 cells and iron binding proteins was investigated. We hereby show that: a) PA exerts a different effect on HepG2 cells compared to BCS, b) co-administration of Cu/PA in HepG2 cells decreased viability, increased ROS, and impaired mitochondrial function c) PA decreased expression of Cp and Tf, and ferroxidase activity. In addition, our preliminary data on WD patients showed a decrease in serum ferroxidase activity in all with a mild increase in ferritin level in some patients.

In our *in vitro* studies we opted to use HepG2 cells, human hepatoma cell line. HepG2 cells have been used in many studies investigating: copper toxicity (Arnal, et al. 2012); ATP7B mediated Cu detoxification (Cater, et al. 2006); and iron uptake (Attieh, et al. 1999). HepG2 cells were reported to maintain the biosynthetic functions of normal hepatocytes such as secretion of ceruloplasmin and albumin (Knowles, et al. 1980), and are considered as well-established models to study Cp regulation (Das, et al. 2007). In our study we tried to mimic WD conditions on HepG2 cells by exposing cells to exogenous Cu and Cu chelators.

Dose dependent treatment of HepG2 cells with Cu revealed an EC50 of around 50 μM that is comparable to serum and tissue concentrations of Cu in WD patients (Aston, et al. 2000). A 50% decrease in viability of HepG2 cells treated with Cu was obtained after 30 min with no notable variation following 3 days of treatment. This unexpected finding is plausible taking into consideration that HepG2 cells have functional ATP7B that maintains Cu homeostasis. Recent article by Arnal et al, showed a 50% decrease in viability of HepG2 cells treated 1 day with Cu; a further decrease of 25% was observed after 5 days (Arnal, et al. 2012).

Hence, the absence of time dependent effect of Cu on HepG2 cells may be attributed to its proangiogenic effect and possible variations in its levels in HepG2 cells. Previous studies reported levels of copper, zinc, iron, and selenium to vary in serum and tumor tissue of cancer patients. Copper concentrations were elevated while zinc, iron, and selenium levels were lowered (Kuo, et al. 2002; Zuo, et al. 2006). Furthermore, the essential role of Cu in pro-angiogenic molecules such as fibroblast growth factor (FGF),

vascular endothelial growth factor (VEGF), and interleukin-1 has been well established and documented (Pan, et al. 2002).

HepG2 cells were exposed to Cu (EC50) for 6 hr then treated with variable doses of PA. Conversely, cells pre-treated with PA for 6 hr, were treated with Cu (EC50). Both methods showed no PA induced protection. However, when HepG2 cells were co-treated with Cu-PA, a 70% decrease in viability and thus a more cytotoxic effect was observed. On the other hand, both Cu treated cells followed by BCS treatment and Cu-BCS co-treated cells exhibited 100% viability showing that BCS exerts a different effect with a possible different mode of action.

To elucidate the underlying mechanism of Cu toxicity, we examined its effect on plasma membrane integrity. A significant change in size and morphology of the cells co-treated with Cu/PA was noted with increase in ROS generation. Using LDH cytotoxicity assay, none of the experimental treatments with each of Cu, Cu/PA, and Cu/BCS released LDH into media. Although this is in discordant with Arnal et al who reported an increase in ROS and LDH release in Cu treated HepG2 and A549 cells (Arnal, et al. 2012), our findings were in line with studies by Jimenez et al who reported no difference in oxidative stress between Cu treated cells and control (Jimenez, et al. 2002).

Starkebaum et al showed in cell free systems that during chelation, PA reduces Cu^{+2} to Cu^{+} generating as well PA radicals. Upon the reduction of Oxygen and superoxide anion Cu^{+} is reoxidized back to Cu^{+2} with concomitant production of H_2O_2 (Figure 11) (Starkebaum and Root 1985). We show a significant increase in ROS generated from HepG2 cells co-treated with Cu/PA (but not Cu/BCS) consistent with those reported by Gupte et al, using breast cancer and leukemia cells (Gupte and Mumper 2007).

$2PSH + 2Cu(II) \rightarrow 2PS^{\cdot} + 2Cu(I) + 2H^{+}$	**Initial reaction: formation of thiyl radical**
$Cu(I) + O_2 \rightarrow Cu(II) + O_2^{\cdot -}$	**Maintenance reaction: Regeneration of Cu(II)**
$Cu(I) + O_2^{\cdot -} + 2H^{+} \rightarrow Cu(II) + H_2O_2$	**Maintenance of radical chain reaction and generation of hydrogen peroxide**
$PS^{\cdot} + PS^{\cdot} \rightarrow PSSP$	**Chain termination**
$2PSH + O_2 \rightarrow PSSP + H_2O_2$	**Overall reaction**

Figure 11: PA oxidation in the presence of Cu. PSH = D-Penicillamine, PSSP = D-Penicillamine disulfide, $PS^{\bullet}$ = D-penicillamine (thiyl) radical (Starkebaum and Root 1985)

Being the first in identifying the effect of Cu-PA co-treatment on HepG2 cells, we investigated cell cycle arrest to better understand its cytotoxic mechanism. The co-administration of Cu-PA induced cell cycle arrest at G2/M phase. An increase from 37% to 63% was obtained from control to Cu-PA treated cells respectively. Similar effects of oxidative stress on phases of cell cycle were reported. Whereas H_2O_2 induced G2/M cell cycle arrest inhibiting osteoblast proliferation, ROS induced G2/M arrest in bronchial epithelial cells (Li, et al. 2009; Longhin, et al. 2013).

Mitochondria, in all aerobic cells are the major source of ROS, including hydrogen peroxide, radicals, and superoxide anion (Figure 12). These can damage protein components of the mitochondrial membrane, which may be prevented by non-enzymatic antioxidants (Glutathione) and enzymes (SOD, Catalase). ROS however are not always dangerous molecules, for they have important physiological roles in cellular senescence, drug-induced tumor cell death, and cell cycle (Fleury, et al. 2002).

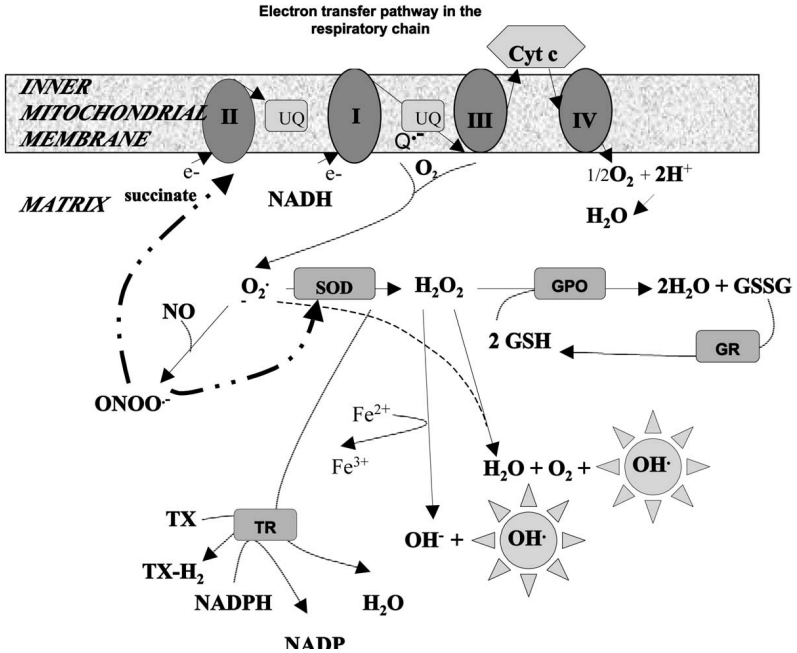

Figure 12: Formation of ROS in mitochondria (Fleury, et al. 2002)

The role of Cu in mitochondria is well known as a key component in electron transport chain enzymes such as cytochrome oxidase, where it mediates electron transfer involving iron-sulfur centers. Cu chelators thus would delete Cu from the cell, consequently affect mitochondrial function. In vitro studies performed on liver mitochondria isolated from LEC rats reported structural and functional mitochondrial modifications occurring at any age, while clinical signs of liver failure were still absent. This was further validated by

the severely impaired enzymatic activities in mitochondrial fractions of clinically apparent Atp7b-/- animals (Zischka, et al. 2011).

Cu-PA treated HepG2 cells showed significant depolarization of mitochondrial membrane potential. Moreover a significant decrease in Complex II (Succinate dehydrogenase) activity was also obtained compared to that of Complex I. Both enzymes provide the entry sites of reducing equivalents into the respiratory chain that ultimately reduces oxygen and generates ATP. Thus, mitochondrial depolarization obtained from PA and BCS treated cells, as well as the decrease in Complexes I and II activities may result from a decrease in Cu levels that disturbs the balance of an ion with high redox potential. Determining intracellular Cu level would verify if Cu disturbance occurred (future studies). Indirect evidence from experiments performed on rats receiving Cu deficient diet have been reported to cause mitochondrial dysfunctions resulting from increased lipid peroxidation (Mehta, et al. 2006).

Cp is a Cu containing protein mainly synthesized in the liver and is the main transporter of Cu in blood. It is a member of the multicopper oxidase family that causes oxidative damage to macromolecules (Mukhopadhyay, et al. 1998); exert oxidizing activity on group of compounds, relating Cp to neurological diseases (Frieden and Hsieh 1976); have peroxidase activity (Cha and Kim 1999); and participate in iron loading onto serum transferrin, implicated thus in iron homeostasis (Okamoto, et al. 1996).

Regulation of Cp synthesis by copper is still unclear, while its regulation with iron has been documented in many experiments (Das, et al. 2007). Rats fed extra Cu, under iron deficient conditions, exhibited increased liver Cu level. Cp mRNA levels in the liver did not change, however serum Cp levels were elevated with enhanced Cp ferroxidase activity

showing evidence of post-transcriptional mechanism (Ranganathan, et al. 2011). An increase in Cp and metallothionein (MT) levels in HepG2 cells treated with Cu was reported (Arnal, et al. 2012). We not only investigated effect of Cu loading on Cp expression, but also effect of Cu deprivation through PA and BCS treatment. Similarly, western blot analysis in our study, show an increase in Cp expression with Cu treated HepG2 cells, while the Cu chelators lowered its expression; with PA being more significant than BCS. Moreover, the increase in copper redox activity, with Cu bound to Pyrrolidine dithiocarbamate (PDTC), increased Cp level in HepG2 and Hep3B cells, an effect that was blocked by BCS (Das, et al. 2007).

Iron, a vital mineral, is required as a structural component in heme/cytochrome proteins and in mediating transfer of electrons for aerobic respiration. Similar to Cu, cellular overload of iron is toxic leading to cell death (Britton, et al. 1994; Siah, et al. 2005). Its incorporation into transferrin is mediated by copper oxidases like hephaestin in the intestine and Cp in the liver. Hence we studied the effect of Cu chelators on Cp ferroxidase activity consequently on the iron binding protein ferritin and serum iron binding protein transferrin. Previous studies reported mice fed copper deficient diet had lowered intracellular copper levels but elevated iron levels in the liver concomitant with a lowered Cp ferroxidase activity. Although Cp levels were not affected in these mice ferritin levels, comprising the major storage form of iron in tissues, were higher than normal (Chen, et al. 2006). HepG2 cells supplied with Cu, or depleted from Fe, resulted in increase in intracellular Cp and transferrin levels, and decrease in Ferritin levels (Fosset, et al. 2009). Similar findings by Mukhopadhyay et al, reported that depletion of iron from HepG2 cells

resulted in a 4 to 5 fold increase in Cp level, that did not vary with Bathocuproine (BC) treatment (Mukhopadhyay, et al. 1998).

In our study, ferritin expression in HepG2 cells treated with Cu, BCS, or PA alone was as expected. However co-treatment with Cu-PA unexpectedly reduced ferritin levels in HepG2 cells. It is reported that oxidative damage to the ferritin shell initiates its degradation and hence its level (Lipiński and Drapier 1997). The obtained increase in ROS level in Cu-PA treated HepG2 cells would explain the immediate decrease in ferritin level in our study. However, ferritin expression could be time dependent and would vary (possibly increase) after a longer exposure to Cu-PA or PA. Further studies differentiating between the mitochondrial and the cytoplasmic ferritin pools may be necessary to understand how ROS generated by Cu/PA mediate and regulate ferritin expression.

Cu is vital for Cp ferroxidase activity. Thus its chelation is expected to reduce its loading into apoCp that is needed for iron incorporation into transferrin. Although the transferrin expression was not quantified, our preliminary data (2 experiments) show qualitative decrease in Tf expression in PA treated cells. This goes in hand with the lowered Cp levels and lowered ferroxidase activity in HepG2 cells treated with Cu chelators. Interestingly, although Cp expression was unaltered with Cu-PA co-treatment, the ferroxidase activity was reduced, along with reduced Tf expression.

To sum up, our in vitro studies show that Cu chelator PA lowered Cp expression, decreased ferroxidase activity, qualitatively decreased Tf, but ferritin level was not affected. Although Cu-PA treatment had no effect on Cp expression, one cannot rule out its effect on ferroxidase activity. Our findings on HepG2 cells with normal ATP7B show a decrease in ferroxidase activity in both PA and Cu-PA treated cells, regardless of Cu level.

Thus, it is plausible to postulate that the normal expression of Cp level is not necessarily commensurate with function. Decrease in Cp ferroxidase activity may be attributed to inappropriate Cu loading on the three Cu sites of apoceruloplasmin. Whether ferroxidase activity in secreted or serum Cp depend on varying levels of incorporated Cu remains to be investigated.

Decrease in ferroxidase activity would affect the oxidation state of iron hence its mobilization outside the cell. Normally, as Fe^{+2} is released from ferritin, it is oxidized by serum Cp to Fe^{+3} prior to its incorporation into Tf. Young et al showed that release of iron from HepG2 cells was enhanced by apo-transferrin and Cp (Young, et al. 1997).

One of the biochemical features in the diagnosis of WD is low Cp level, although some patients may have normal levels. Patients are usually managed with life-long treatment using Cu chelators. This would increase excretion of Cu, decrease Cu incorporation in various cuproproteins, and lower Cu dependent ferroxidase activity of Cp. Golan et al determined oxidase activity of Cp in 2 WD families where patients had abnormal low Cp Oxidase activity (Gollan, et al. 1977). This would impair iron loading into serum transferrin and consequently deposit iron in the liver with subsequent increase in ferritin level. Increase in iron levels may thus either result from an increase in iron absorption, or a decrease in efflux. Furthermore, the increase in iron may be iatrogenic as reported in WD patients who had normal iron level before PA treatment (Schilsky 2001).

The physiology of iron metabolism in WD patients is largely unknown, and the data on presence and progression of iron accumulation is scarce (Medici, et al. 2007). In our study we have recruited 5 patients belonging to two Lebanese families. All patients were diagnosed with WD based on clinical and genetic criteria. All were homozygous for C

insertion in exon 8 (P2, P3, P4, P5), except for one with missense mutation in exon 18 (P1). Serum Cp ferroxidase activity was low in all patients compared to normal range. In addition serum ferritin was mildly elevated in 2 patients out of 5 (P2 and P3). One patient (P1), who is currently on Zinc, (initially on PA, discontinued because of side effects) was recently diagnosed with WD, and had very low ferroxidase activity. One heterozygous patient who was also tested had ferroxidase activity between the homozygous and normal values. Whether homozygosity or heterozygosity of the ATP7B have any bearings on the ferroxidase activity remains to be established by recruiting more patients who are either homozygous, heterozygous, or carriers of disease causing mutations.

In one retrospective study, four male compound heterozygous WD patients with hepatic manifestations, ages of onset between 16 and 23, and on PA treatment were examined over a period of 3 to 8.5 years. Histochemical analysis of Cu (by Rhodamine) and iron (by Perls' staining) and biochemical assays of liver biopsy performed pre and post PA treatment showed: a) further decrease in serum Cp level, b) lower Cp ferroxidase activity, c) decreased liver Cu content, and d) Fe accumulation in the liver (Shiono, et al. 2001). In another study, Luca et al described a WD patient who after 15 years of combination treatment with PA and Zinc had massive hepatic iron deposition, assessed by a liver biopsy, which would have been misdiagnosed with genetic hemochromatosis (Schilsky 2001). Moreover, histochemical Fe and Cu staining and X ray microanalysis was performed on 16 WD patients of mixed genotypes, pre and post their treatment with PA. 2 patients displayed iron dominant combined overloading of the two metals, while 4 exhibited isolated iron loading and reduced Cu levels (Hayashi, et al. 2013).

To sum up, our *in vitro* and clinical studies, provide preliminary data on changes in expression level of Cp and ferritin as well as decrease in Cp ferroxidase activity in WD patients treated with PA. In addition, our findings provide preliminary evidence of possible therapeutic complications of the life-long treatment with PA. Establishment of new modalities in WD treatment such as non-invasive monitoring of Fe level using MRI and/or recommending Fe chelators should be considered. Mak et al recommended that every patient should have baseline Cp ferroxidase activity measured before the start of treatment. To avoid excessive Cu chelation, serum free-Cu concentration should not get lower than the lower limit of the age specific reference range (Mak and Lam 2008).

Future Studies

We plan to investigate

- Intracellular Fe and Cu levels in HepG2 cells treated with Cu and Cu chelators using ICP.
- Effect of Cu/PA treatment on isolated primary hepatocytes and immortalized normal Hep3B cells.
- Effect of Cu and Cu chelators on expression and transcription of ceruloplasmin, ferritin, and transferrin after 48 and 72 hr of treatment.
- Fractionation of HepG2 cells to determine effects of Cu, PA, and Cu/PA on expression levels of a) mitochondrial ferritin verses cytoplasmic and b) GPI-anchored Cp and Cytoplasmic.

- MRI of the liver of WD patients is a non-invasive method to verify presence of iron in the liver.
- A greater number of WD patients needs to be recruited in this study. Patients taking medication other than Penicillamine like Trientine or Zinc will be recruited to compare the ferroxidase profile between these groups.
- LEC rats placed on Penicillamine treatment can be monitored for fluctuations in levels of iron binding proteins in the serum, as well as iron levels in the liver via biopsy.

Limitations

- HepG2 cells are one of the most extensively used cell lines in WD research, however ATP7B in these cells is not mutated, hence intracellular Cu overload that characterizes WD doesn't occur.
- Maintaining the cells in medium enriched with Cu was not sustained more than 5 days, since cells failed to adhere and reach confluence.
- We had no estimate of the levels of iron saturation and iron binding proteins in the patients prior to their PA treatment, and hence it was difficult to assess the change that had occurred in their levels with chelation.
- The number of patients was limited to 5.

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