CYTOTOXIC MECHANISMS FOLLOWING THE OVERLOADING OF ISOLATED RAT HEPATOCYTES WITH IRON VERSUS COPPER

by

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ABSTRACT

The administration of ferric nitrilotriacetate ($Fe^3(NTA)_2$) or cupric nitrilotriacetate ($Cu^{2+}(NTA)_2$) to mice or rats causes an overload of iron and copper in the liver and kidney which can lead to hepato and nephrotoxicity. The molecular cytotoxic mechanisms involved have been assumed to be similar. In the following, isolated rat hepatocytes were incubated with $Fe^{3+}(NTA)_2$ or $Cu^{2+}(NTA)_2$ and different cytotoxic mechanisms were found.

1. $Fe^{3+}(NTA)_2$ -induced lipid peroxidation before cytotoxicity occurred. Cytotoxicity was prevented if lipid peroxidation was prevented with antioxidants (DPPD and BHA). However, added catalase and superoxide dismutase (SOD) did not affect cytotoxicity. $Cu^{2+}(NTA)_2$ was 6-fold more toxic than $Fe^{3+}(NTA)_2$ but caused much less lipid peroxidation. $Cu^{2+}(NTA)_2$ -induced cytotoxicity was also not prevented by antioxidants but was prevented by catalase and SOD. This suggests that $Fe^{3+}(NTA)_2$ cytotoxicity can be attributed to lipid peroxidation whereas $Cu^{2+}(NTA)_2$ cytotoxicity can be attributed to reactive oxygen species.

cytotoxicity whereas DTT did not affect $Fe^{3+}(NTA)_2$ cytotoxicity. $Cu^{2+}(NTA)_2$ and $Fe^{3+}(NTA)_2$ cytotoxicity were markedly enhanced in GSH-depleted hepatocytes.

3. $Cu^{2+}(NTA)_2$ and $Fe^{3+}(NTA)_2$ cytotoxicity were similar in that the mitochondrial membrane potential decreased before cytotoxicity ensued. Cytotoxicity was also more pronounced at low oxygen concentrations. This suggests that the ATP depletion associated with the loading of hepatocytes with Fe^{3+}/ Cu^{2+} results from mitochondrial toxicity. The loss of mitochondrial membrane potential by $Fe^{3+}(NTA)_2$, however was prevented by antioxidants, whereas $Cu^{2+}(NTA)_2$ toxicity was prevented by catalase.

4. Clinically, hepatic Fe^{3+}/ Cu^{2+} overload is treated by chelation therapy with desferoxamine or penicillamine respectively. It was found that the ferric chelator, desferoxamine prevented both $Fe^{3+}(NTA)_2$ - and $Cu^{2+}(NTA)_2$ -induced hepatocyte cytotoxicity. $Cu^{2+}(NTA)_2$ -induced cytotoxicity was also prevented by ammonium tetrathiomolybdate much more effectively than by penicillamine.

5. The cytotoxic mechanisms of the Cu^{2+} complexes of o-phenanthroline (OP) or neocuproine (NC) previously shown to be cytotoxic to tumor cells were also compared. Recently, these complexes have been proposed for AIDS therapy as HIV-1 integrase inhibitors. It was found that $Cu^{2+}(OP)_2$ cytotoxicity unlike $Cu^{2+}(NC)_2$ hepatocyte cytotoxicity was associated with extensive oxygen activation and lipid peroxidation presumably because its significantly lower redox potential favored oxygen activation.

However, antioxidants did not prevent cytotoxicity. Furthermore, $Cu^{2+}(OP)_2$ cytotoxicity ensued with little GSH depletion whereas $Cu^{2+}(NC)_2$ cytotoxicity ensued following extensive GSH depletion as a result of GSH efflux. However, both complexes were similar in that cytotoxicity was prevented or delayed by dithiothreitol, DMSO, and catalase, and markedly enhanced if hepatocyte GSH was depleted beforehand. Thus although these complexes cause different changes in hepatocytes, the reactive intermediates resulting in cytotoxicity appear to be reactive oxygen species formed intracellularly by Cu^{2+} released from the complexes.

DEDICATIONS

This thesis is dedicated to :

my Father and Mother

my Grandmother

my Brother

who have outpoured love

and support and understanding

throughout all my life

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SUMMARY OF ABBREVIATIONS

ABBREVIATION	NAME
ALT	Alanine transaminase
AST	Aspartic acid transaminase
BCS	Bathocuproine disulfonate
BHA	Butylated hydroxyanisole
BPS	Bathophenanthroline disulfonate
BUN	Blood Urea Nitrogen
Cp	Ceruloplasmin
Cox	Cytochrome c oxidase
DMSO	Dimethyl sulfoxide
DPPD	N,N'-Diphenyl-1,4-phenylenediamine
DTT	Dithiothreitol
EDTA	Ethylenediaminetetracetic acid
ED_{50}	Effective dose required to kill 50% of the cell population at 2 hours
EGTA	Ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
ER	Endoplasmic reticulum

GSH	Reduced glutathione
GSSG	Oxidized glutathione
γ-GTP	γ-Glutamyltranspeptidase
α-KG	α-ketoglutarate
HPLC	High Performance Liquid Chromatography
MDA	Malondialdehyde
MT	Metallothionein
Mt	Mitochondria
NC	Neocuproine (2,9-dimethyl-1,10-phenanthroline)
NTA	Nitrilotriacetic acid
NTBI	Non-transferrin bound Iron
OP	1,10-phenanthroline
P450	Cytochrome P450
ROS	Reactive oxygen species
SD	Sprague-Dawley
SE	Standard Error
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TTM	Ammonium tetrathiomolybdate
TGR	trans-Golgi reticulum

SUMMARY OF FIGURES

Chapter 1

General Introduction

Oxygen radicals and other oxygen-derived species have been implicated as important causative agents of aging and degenerative diseases such as cancer, multiple sclerosis, Parkinsonism, autoimmune disease and senile dementia. Oxygen radical species and lipid peroxidation have also been implicated in the toxic mechanisms of a wide range of xenobiotics, including paraquat, adriamycin, ethanol, alloxan, tetrachloromethane, daunomycin and many more.

Transition metals, namely iron and copper, seem to be involved in this oxidative stress cytotoxicity as toxicity was prevented by certain transition metal chelators which presumably act by preventing hydroxyl radical formation from Cu^{2+}/ Fe^{3+} catalyzed reaction with hydrogen peroxide (better known as the Fenton or Haber-Weiss reaction) (Gutteridge and Halliwell, 1985).

1.1. REACTIVE OXYGEN SPECIES (ROS)

1.1.1. Formation of superoxide anion radical

Oxygen metabolism by biological processes can involve the formation of small amounts of $\bullet O_2^-$, H_2O_2, and $\bullet OH$ and various unstable oxidized lipids. The electron

transport chain of mitochondria is one of the major sites for the production of ROS under physiologic conditions. Under normal conditions, cytochrome c oxidase reduces O_2 consumed by cells to form H_2O by the sequential transport of 4 electrons. However, it has been estimated that 1-2% of the mitochondrial electron flow reduces O_2 to $\bullet O_2^-$ (Sandhu et al., 1992).

Superoxide anion radical is produced *in vivo* also during the respiratory burst of phagocytotic cells and plays a crucial role in the bactericidal function of phagocytes. Various enzymes including amine oxidase, cytochrome P450, xanthine oxidase and prostaglandin synthase also produce $\bullet O_2^-$.

1.1.2. Formation of hydrogen peroxide

Upon reduction of molecular oxygen to $\bullet O_2^-$, it dismutates to H_2O_2 either spontaneously or catalyzed by superoxide dismutase (SOD). H_2O_2 readily permeates the plasma membrane unlike the superoxide anion radical. In several experiments which expose cells to $\bullet O_2^-$ generating systems, the "cytotoxic agent" has been identified as H_2O_2 since this species can permeate the cell. Furthermore, SOD was ineffective compared to catalase in protecting the cells. Nonetheless, one should not fully assume that H_2O_2 itself is the ultimate toxic agent.

1.1.3. Formation of Hydroxyl radical (•OH)

The *in vitro* formation of the •OH radical has been implicated in the classic Haber-Weiss reaction catalyzed by transition metals (M^{n+}), which can be Ti(III), Cu(I), Fe(II) or Co(II). The transition metals most involved in •OH generation *in vivo* appear to be iron and to a lesser extent, copper (Halliwell and Gutteridge, 1984). The following depicts the metal-catalyzed Haber-Weiss reaction:

$$Fe^{3+}\ (Cu^{2+}) + \bullet O_2^- \Longrightarrow Fe^{2+}\ (Cu^{1+}) + O_2$$

$$Fe^{2+}\ (Cu^{1+}) + H_2O_2 \Longrightarrow Fe^{3+}\ (Cu^{2+}) + \bullet OH + OH^-$$

$$\bullet O_2^- + H_2O_2 \xrightarrow[\text{catalyst}]{\text{Fe or Cu}} \bullet OH + OH^- + O_2$$

However, whilst •OH is formed at an acid pH, the cytotoxic oxidant formed at a physiological pH may be $Fe^{2+}O$ or $Cu^{1+}O$ formed as follows (Liochev and Fridovich, 1994; Petering et al., 1992):

$$HOOH + Fe^{2+} \text{ or } Cu^{2+} \leftrightarrow H^+ + Fe^{1+}OOH \text{ or } Cu^{1+} + \bullet OOH$$

$$Fe^{1+}OOH + \bullet OOH \leftrightarrow Fe^{2+}O + HO^-$$

Alternatively, $Cu^{1+} + HOOH \leftrightarrow Cu^{1+}O + H_2O$

$$Fe^{2+}O \text{ or } Cu^{1+}O + H^+ \leftrightarrow Fe^{3+}\text{-}OH \text{ or } Cu^{2+}\text{-}OH \leftrightarrow Fe^{3+} \text{ or } Cu^{2+} + \bullet OH$$

1.2. Destructive Effects of ROS

Figure 1 is a cartoon depicting the role of various reactive oxygen species (ROS) and their destructive effects towards cellular membranes, organelles and chromatin (Hooper, 1989). It indicates the predominance of H_2O_2 as the species that can permeate the cell and give rise to more reactive species within the cell by reacting with transition metals. Superoxide anion radicals have been discovered to reductively release protein-bound iron from endogenous iron-sulfur proteins or aconitase (Gardner and Fridovich, 1991; Fridovich, 1994). The generation of ROS from the Haber-Weiss reaction causes site-specific disruption to plasma membrane, nuclear membrane, chromosomes and other organellar structures. The reaction of ROS with phospholipids, also generates lipid peroxyl radicals (LOO•) , which have short half-lives (Grisham, 1992) and perpetuate the disruption of cellular or organellar membranes, depending on the target site of ROS generation.

Superoxide anion radicals may also be cytotoxic independently of the Haber-Weiss reaction by reacting with nitric oxide (NO•) to yield peroxynitrite ($ONOO^-$), which is a powerful oxidizing agent similar to •OH (Beckman et al., 1990).

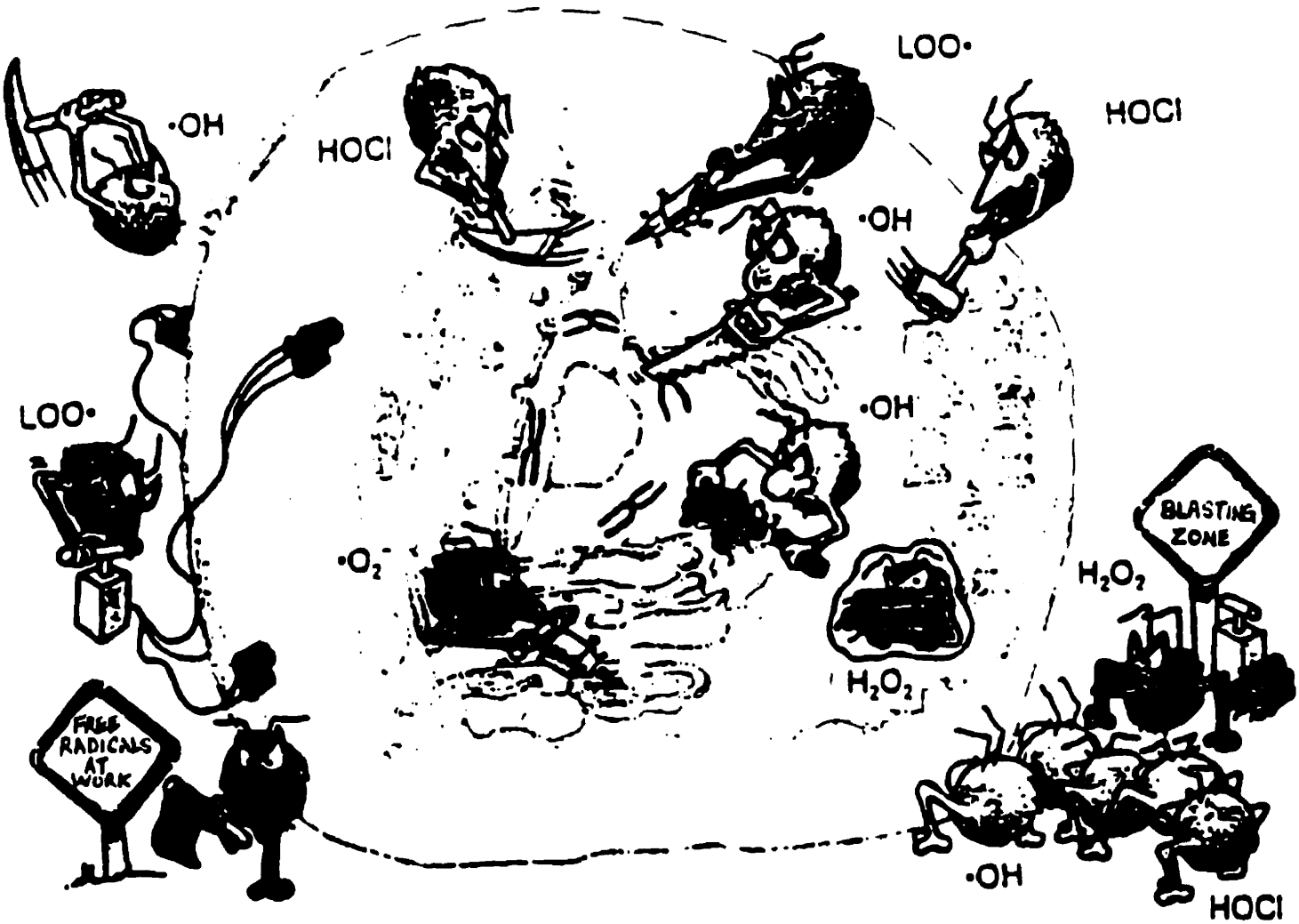


Figure 1.1 : Cartoon of the varied destructive actions of ROS on cellular, organellar and chromatin structures (Hooper, 1989)

1.3. Copper and Iron in Biological Systems

1.3.1. Iron: Metabolism, uptake and toxicity

It is the second most abundant metal on earth after aluminium and it is abundant in biological systems. The ferric ion has a redox potential of approximately 1V (Crichton, 1991) and plays a significant role in biological systems. It forms essential active centers of certain enzymes, including ribonucleotide reductase, aconitase, cytochromes as well as heme proteins, namely hemoglobin, myoglobin and cytochrome P450s.

Adult men and women maintain a constant level of approximately 55 and 45 mg iron/kg body weight respectively. About 60-70% of the total iron in the body is found in hemoglobin, while myoglobin, cytochromes and other iron-containing enzymes comprise a further 10%. The remaining 20-30% is distributed between two storage proteins : ferritin and the lysosomal degradation product, hemosiderin. Transferrin, which is an extracellular transport protein, accounts for only 0.1-0.2% of the total body iron. There have also been recent questions about the nature and role of intracellular iron consisting of small and soluble iron complexes, also known as the low-molecular mass iron pool (Fontecave and Pierre, 1993).

Iron is available from dietary sources (1-3mg/day). Under normal conditions, this amount is lost in the bile, urine, shed intestinal cells and from the skin. Absorption, however, occurs through the gastrointestinal mucosa and iron is transported in the blood bound to transferrin. Transferrin is an 80kDa glycoprotein, with two high-affinity ferric binding sites (stability constant about 10^{20}) (Fontecave and Pierre, 1993).

Two mechanisms for the cellular uptake of iron-bound transferrin have been identified: The first is a receptor-mediated endocytotic mechanism. This mechanism requires the binding to a specific receptor on the cell surface, resulting in internalization of the transferrin-iron complex into an acidic non-lysosomal vesicle. Mobilization of iron is facilitated by lowered pH; however, this mechanism is receptor-dependent and does not apply to all cell types (Huebers and Finch, 1987). In hepatocytes, the alternative transport mechanism involves the binding of iron-transferrin to a specific receptor on the plasma membrane, preceeding the reduction of iron by a specific membrane diferric-transferrin reductase. This process is followed by an Fe(II) transport system translocating the iron into an intracellular space prior to incorporation of the ferric ion into ferritin. One molecule of ferritin can store up to 4500 iron atoms, mainly in the form of inorganic oxyhydroxide, together with some phosphate (Crichton, 1991). The ferritin molecule reduction potential is -230mV at pH 7.0 and may vary depending on the iron content of the core (De Silva and Aust., 1992).

The redox potentials of hemoproteins and cytochromes and iron-centered enzymes are determined by the nature and steric arrangement of the surrounding ligands. Enzymes which have iron as redox active sites shield the reactive metal from the external environment and prevents the unnecessary side reactions between reactive intermediates and membrane phospholipids (Silva and Aust, 1992).

Iron in its reduced state is reactive towards oxygen. The following scheme shows the reactivity of ferrous iron with oxygen in the Fenton reaction upon reduction by either enzymatic systems or by small molecules.

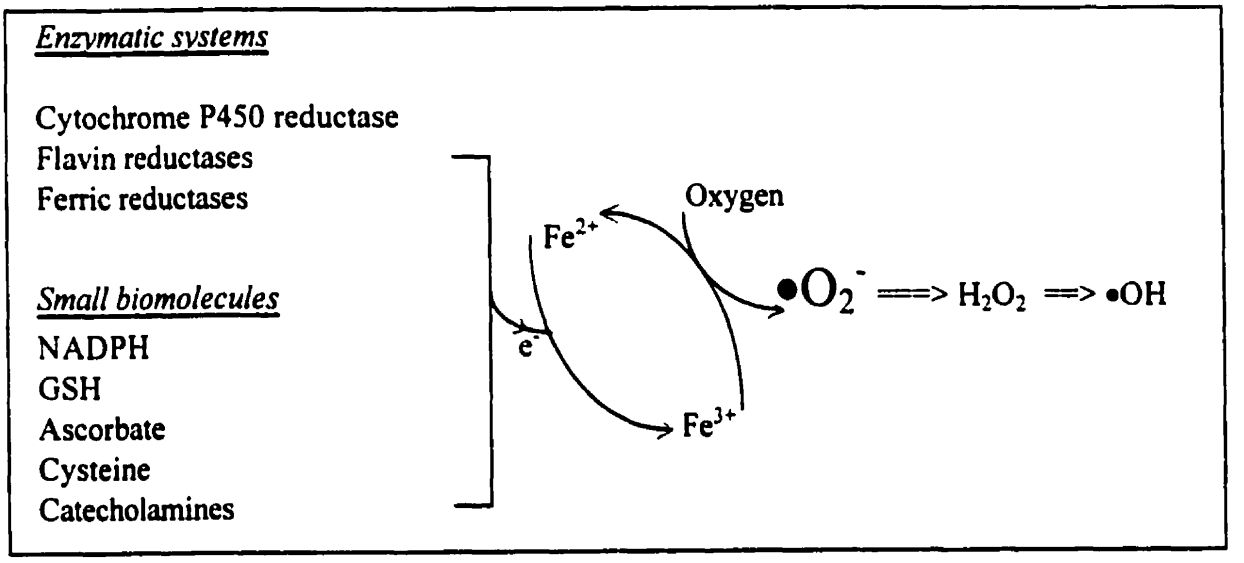


Figure 1.2: The biological contribution and reactivity of iron catalyzed oxygen activation

1.3.2. Mechanisms and consequences of Iron Overload

Tissue iron overload eventually occurs when plasma iron concentration exceeds the 3-fold total iron binding capacity of transferrin. This arises when there is excessive dietary iron intake or blood transfusions (thalassemia therapy). This also arises in inherited diseases, namely idiopathic haemochromatosis, where there is an excessively high gut absorption of iron, or congenital atransferrinemia, where there is a total absence of circulating transferrin .

The low-molecular mass iron pool in the liver/kidney consists of iron bound to negatively charged ligands, including citrate, ammonium-citrate, nitrilotriacetate, or adenosine diphosphate (ADP) (Lesnefsky, 1994). Iron bound to these ligands is also known as non-transferrin bound iron (NTBI). These ligands enhance the redox cycling of iron and catalyze oxidative damage. In tissue iron overload, the levels of ferritin and hemosiderin will increase and overwhelm the cell's capacity to store excess cytosolic iron which predisposes the cell to oxidative injury.

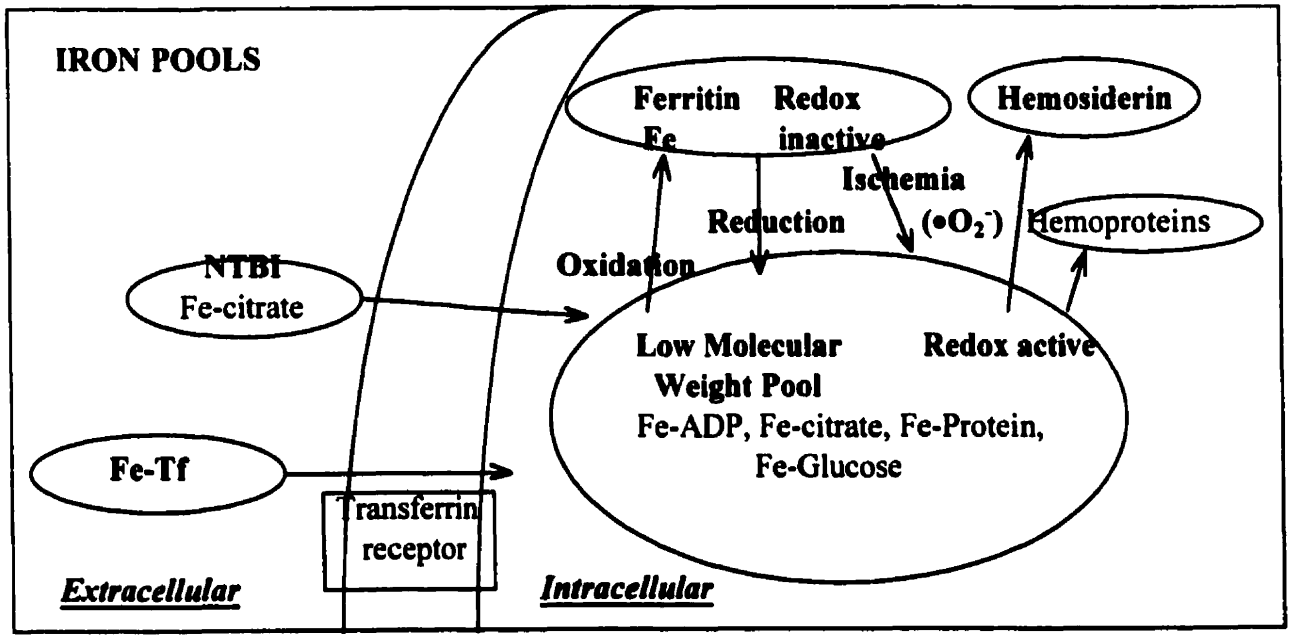


Figure 1.3: Schematic summary of cellular uptake and distribution of iron intracellularly from cellular storage proteins and the low-molecular mass iron pool (Lesnefsky, 1994).

1.3.3. Copper: Metabolism, uptake and toxicity

Total body copper in healthy adults (70kg) ranges between 80-150mg. The concentration of copper in the liver (8-50%) and relative contribution to total copper in the body may vary significantly depending on the stage of human growth. Fetal livers contain between 50 and 60% of total body copper. Adult livers, on the other hand, contain 5-fold less copper than fetal livers (Luza and Speisky, 1996). Copper absorption occurs in the gastrointestinal tract, primarily in the stomach and jejunum and copper is transported through the portal circulation system mostly bound to albumin and transcuprein (Linder and Azam, 1996). Removal of copper from the liver results either in retention by parenchymal cells, or is excreted into the bile or released back into the plasma. Therefore, in the posthepatic circulation, ceruloplasmin, albumin, transcuprein and some copper-binding amino acids (L-histidine) contribute to the transport and redistribution of copper in circulating plasma (Gubler et al., 1953).

In rat hepatocytes, copper is heterogeneously distributed among subcellular fractions. It has been estimated by Smeyers-Verbeke et al. (1992) that the normal average intracellular distribution of copper is nucleus (27%), mitochondria (7%), RER (7%), SER(3%) and cytosol (54%) respectively. In the copper overloaded liver, however, copper becomes associated with particulate fractions such as lysosomes , nuclei and mitochondria (Ryder and Deutschen, 1978; Gregoriadis and Sourkes, 1967).

Relative to other physiologically occurring divalent metals, copper reacts especially well with proteins, binding to the amine, thiolate and carboxyl ligands present

in these macromolecules (Cousins, 1994). Cellular homeostatic mechanisms are designed to strictly regulate the intracellular availability of copper and copper-binding ligands. Upon entry into hepatocytes, ionic copper is rapidly bound to low-molecular weight cytosolic ligands including metallothionein, glutathione and other higher-molecular weight peptides (Bremner, 1987). Some are temporary carriers, which supply copper for the necessary metabolic requirements in the cell (SOD and cytochrome c oxidase), whereas other carriers serve as storage ligands. Copper is preferentially accumulated in the lysosomes following its binding to metallothionein (Johnson et al., 1981). Metallothionein has a molecular mass of approximately 6,500 Da and has a high capacity to bind copper up to 10g atoms/mol. It contains 30% cysteine residues, and all are involved in storing copper (Kagi and Nordberg, 1979). Each copper-metallothionein complex contains 11-12 atoms of copper, 6 bound to an amino-terminal portion while the remainder are bound to the carboxyl-terminal portion. Copper can be released from metallothionein by oxidation of the bound metal or oxidation of thiol ligands (Nartey et al., 1987).

Figure 1.4 depicts the possible pathways for copper uptake by the cell and the involvement of biomolecules in distribution of cytosolic copper to apoproteins that require copper as a cofactor or a redox active center. Both GSH and MT are known Cu(I) ligands responsible for facilitating the incorporation of copper into SOD1 in the cytosol, peroxisomes (Crapo et al., 1992), mitochondrial cytochrome c oxidase, lysyl oxidase in the Golgi and secretory organelles (Kulvaniemi et al., 1986). Cu-MT is found in the cytosol, nucleus and lysosome fractions (Jannsen et al., 1974).

Genetic diseases resulting in tissue copper overload include I) male and female homozygous toxic-milk mice and II) Wilson's disease. In the latter case, increased copper in hepatocytes initially accumulates in the cytoplasm prior to deposition in lysosomes. Liver MT levels are greatly elevated to store the free cytosolic copper, whereas the rate of biliary excretion of copper is decreased. Consequently, increased hepatocyte copper accumulation is observed. The consequences of tissue copper overload include chronic liver disease with cirrhosis, renal tubular dysfunction, pigmented corneal rings (Kayser-Fleischer rings) and neurologic disorders (behavior disturbances, dysarthria and movement disorders) (Sternlieb, 1990).

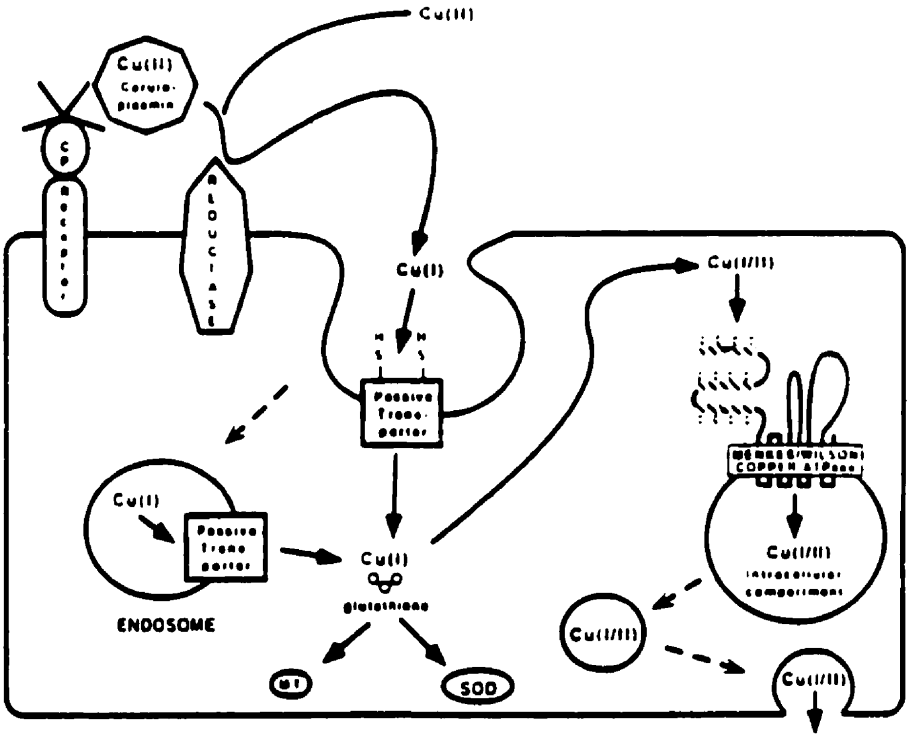


Figure 1.4: Model depicting copper transport involving either the ceruloplasmin-mediated or free copper transport pathway and intracellular distribution of copper to apoproteins by MT and GSH (Vulpe and Packman, 1995)

1.3.4. GENETIC BASIS FOR COPPER AND IRON TOXICITY

Two inherited disorders in copper metabolism are Wilson's disease and Menkes syndrome. Despite both having distinct clinical symptoms, the primary disorder results from abnormal copper transport. Wilson's disease manifests during late childhood with a marked increase in liver and brain Cu^{2+} levels. This results in hepatic cirrhosis and neurologic degeneration, primarily in the basal ganglia (Sternlieb and Steinberg, 1979). It is a recessive autosomal disorder that results from defective biliary copper excretion. Menkes syndrome is an X-linked disease that decreases placental transport and the gastrointestinal copper absorption resulting in severe deficiency of copper dependent enzymes (Kodama, 1993). Victims of Menkes syndrome suffer from progressive mental deterioration, hypothermia, failure to grow and connective tissue abnormalities that cause mortality during early childhood (Kodama, 1993).

Genes responsible for Menkes syndrome and Wilson's disease have been cloned and have been identified with transmembrane proteins homologous to cation-transport P-type ATPases (Tanzi et al., 1993; Yamaguchi et al., 1993). The Menkes and Wilson's P-type ATPases are 65% homologous and contain an amino-terminal domain with 6 repetitive Cys-X-X-Cys motifs (X represents any amino acid). The Menkes gene product is localized in most tissues except the liver, while the Wilson gene product that is expressed predominantly in the liver. Both genes are remarkably conserved with specific sequences found also in other cation-transport proteins. This is illustrated in the carboxy-terminus, which contains a phosphorylation domain (Asp-Lys-Thr-Gly-Thr) together

with an invariable Asp residue located on all P-type ATPases. The ATP-binding domain (Gly-Asp-Gly-Ile-Asn-Asp) is another highly conserved domain. The conservation of another particular domain between the two mentioned domains is the Ser-Glu-His-Pro sequence. Apparently, >25% of mutations characterized in Wilson's disease involves a missense mutation (His to Gln) at this site (Petrukhin et al., 1994; Thomas et al., 1995). In Menkes syndrome, the severity of the disease is dependent on variations due to splicing defects in distinct regions (Kaler et al., 1994; Das et al., 1994). Fusion proteins generated from polyclonal antisera have recently been used to define the intracellular location of these ATPases (Yamaguchi et al., unpublished). Results have implied that Menkes and Wilson P-type ATPases are localized within the late secretory pathway in cells (Figure 1.5) (Harris and Gitlin, 1996). Here, the P-type ATPase transports copper from the cytosol into the secretory pathway, making it available for incorporation into ceruloplasmin. The copper-ceruloplasmin complex is then sequestered into endosomes for transport into the bile (Harris and Gitlin, 1996).

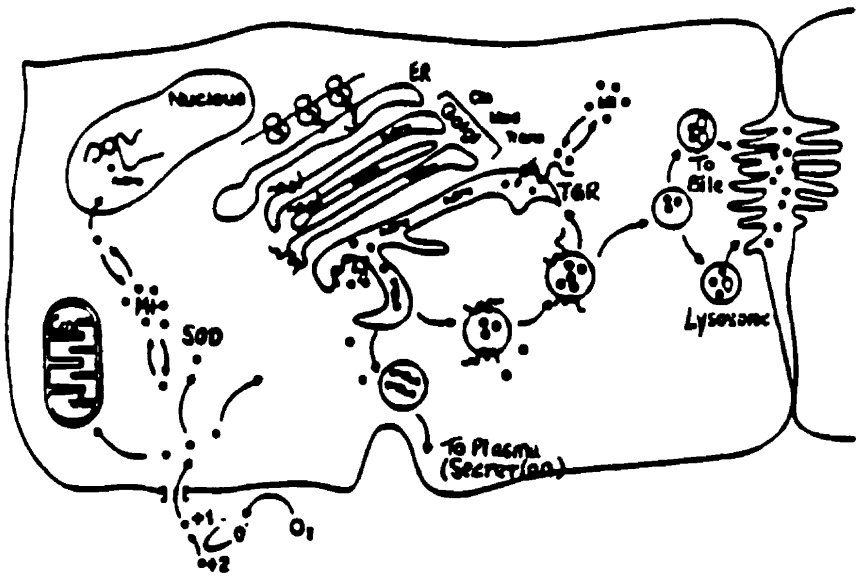


Figure 1.5: Copper metabolism modelled in hepatocytes identifying role of Wilson P-type ATPase in copper sequestration in the late endosomal compartment (Harris and Gitlin, 1996)

A recent discovery in the genetic basis of iron toxicology has linked the role of ceruloplasmin (Cp) to ferrous iron and its release from ferritin as well as the uptake of ferric iron and transport by transferrin. Aceruloplasminemia was first investigated in patients diagnosed with Wilson's disease who suffered from diabetes, dementia, basal ganglia symptoms and decreased levels of serum Cp. However, these clinical symptoms were more characteristic of a subcortical dementia related to Parkinson disease. Liver biopsies even revealed normal copper concentrations but elevated iron stores (Hiyajima et al., 1987; Logan et al., 1994; Morita et al., 1995). These clinical symptoms were also distinct from hemochromatosis. Molecular genetic analyses of patients has revealed mutations in the Cp gene that affect the open reading frame of the gene (Harris et al., 1995; Yoshida et al., 1995; Takahashi et al., 1996). Figure 1.6 proposes the role of Cp in iron metabolism due to its essential role as a copper oxidase (Dancis et al., 1994). In the suggested mechanism, there is excessive ferrous iron accumulating in glial cells, ECF and CSF that cause neurodegeneration from free radical injury (via Haber-Weiss reaction) as well as iron deficiency in neurons as a consequence of defective delivery of transferrin ferric iron (Tomac et al., 1995; Beck et al., 1995; Oppenheim et al., 1995).

Both Wilson's disease and aceruloplasminemia are the consequences of genetic mutations that portray the toxicology of copper and iron overload respectively. Both genetically-based diseases are involved in the altered transport, uptake and efflux of the essential metals that ultimately result in excessive accumulation of copper and iron in the liver and brain respectively.

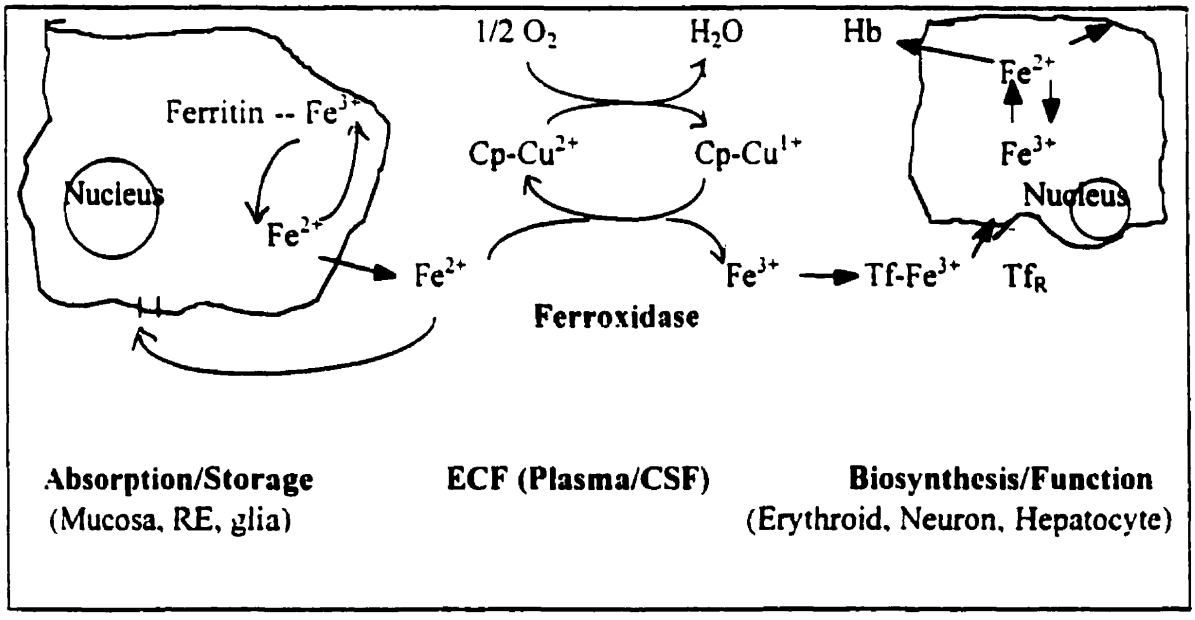


Figure 1.6: Proposed role of Cp as a ferroxidase and the potential sites of injury due to aceruloplasminemia (Harris and Gitlin, 1996).

1.4. CHELATION THERAPY FOR COPPER/IRON OVERLOADING

It has been shown that under iron/copper overloading, free iron/copper is detected in the circulation and is highly capable of promoting cellular oxidative stress and saturating the physiological homeostatic controls of these metals, namely transferrin and ceruloplasmin, respectively. All these effects participate in tissue damage (liver, kidney, spleen and heart), cardiac abnormalities, skeletal and cranial defects and subsequent death (Fontecave and Pierre, 1993).

Treatment of iron overload has depended successfully for the past 20 years on desferoxamine mesylate, a potent ferric chelator. It was isolated as the iron chelate from *Streptomyces pilosus* and was treated chemically to yield the metal-free ligand.

Desferoxamine has an exceptionally high affinity for ferric iron ($K_a = 10^{31}$) and removes iron from hemosiderin and ferritin, and to a lesser extent from transferrin. However, it does not remove iron from hemoglobin and cytochromes (Goodman and Gilman, 1993). Current research has also revealed interest in several new iron chelators, namely hydroxypyridinones which have shown potential as desferoxamine substitutes in iron overload therapy (Dobbin and Hider, 1990).

In copper overload therapy, penicillamine has been longest in use. It acts by reductive chelation, followed by urinary excretion of copper. It is an aggressive anticopper drug which produces a rapid initial negative copper balance, which gradually decreases as the mobilizable copper pool shrinks (Walshe, 1956). Trientine (triethylenetetramine), another copper chelator applied in overload therapy also increases urinary excretion of copper (Walshe, 1982). It is used for patients intolerant of penicillamine. Both penicillamine and trientine are fast-acting, but cause moderate to high incidence of neurologic disorders and autoimmune disorders in some patients (Brewer and Yuzbasiyan-Gurkan, 1992; Brewer et al., 1987) possibly because the copper penicillamine and trientine complexes can still redox cycle and activate oxygen. The most recent therapy in clinical trial is ammonium tetrathiomolybdate, which blocks intestinal absorption of copper and detoxifies blood copper by forming a tripartite complex with albumin and copper (Brewer et al., 1994). Tetrathiomolybdate reacts with copper to form heterobimetallic complexes through -Mo-S-Cu- clusters (Suzuki et al., 1992)

1.5. RESEARCH OBJECTIVE

The first objective of this thesis was to investigate the possible differences between copper and iron overload toxicity mechanisms in isolated rat hepatocytes as the liver is generally the main target of metal accumulation and intoxication. The second objective was to study the cytotoxic mechanisms of anticancer copper:phenanthroline derivative complexes.

1.6. HYPOTHESES

I) Because both copper and iron are redox-active and activate oxygen via the Haber-Weiss reaction, cytotoxicity resulting from overloading hepatocytes with copper and iron should have common cytotoxic mechanisms and a unifying mechanism leading to cell death.

II) Lipid soluble phenanthroline derivatives which form redox-active copper complexes should pursue different cytotoxic mechanisms from those derivatives that form redox-inert copper complexes after they permeate the hepatocyte cell membrane. However, similar cytotoxic mechanisms could be expected if copper is released intracellularly from the complexes.

Chapter 2

Materials and Methods

2.1. Chemicals

Ferric ammonium citrate, cupric sulfate, sodium nitrilotriacetate acid (NTA), butylated hydroxyanisole (BHA), antimycin A, trypan blue, hydrogen peroxide, reduced glutathione, iodoacetic acid, thiobarbituric acid, catalase (EC 1.11.1.6), trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64), leupeptin, superoxide dismutase (EC 1.15.1.1), ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), ethylenetetradiaminetetraacetic acid (EDTA), trichloroacetic acid, glutamine, Urea nitrogen kit and transaminases (ALT and AST) diagnostic kits, sodium pentobarbital and heparin were obtained from Sigma Chemical Co. (St. Louis, MO).

1-Bromoheptane, N,N'-diphenyl-1,4-phenylenediamine (DPPD), bathophenanthroline disulfonate, bathophenanthroline, o-phenanthroline, neocuproine, bathocuproine disulfonate, bathocuproine, dithiothreitol (DTT), ruthenium red, rhodamine 123, sodium cyanide and fluoro-2,4-dinitrobenzene (FDNB) were obtained from Aldrich Chemical Company Inc. (Milwaukee, WIS). Collagenase (from *Clostridium histolyticum)*, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), and bovine serum albumin were obtained from Boehringer-Mannheim (Montreal, PQ).

Desferoxamine was a gift from Ciba-Geigy Canada Ltd. (Toronto, ON). HPLC grade solvents were obtained from Caledon (Georgetown, ON).

2.2 Animal Treatment and Hepatocyte Preparation

Adult male Sprague-Dawley rats, 250-300g, were obtained from Charles River Canada Laboratories (Montreal, PQ.), fed *ad libitum* and were allowed to acclimatize for one week on clay chip bedding. Freshly isolated hepatocytes were chosen as the intact cell model for studying the relative cytotoxic mechanisms of various copper complexes and to compare differences between iron and copper overload toxicology (Carini et al., 1992, Moldeus, 1978).

The hepatocytes were prepared by collagenase perfusion of the liver as described by Moldeus (Moldeus et al., 1978). Damaged cells, debris, and Kupffer cells were removed by centrifugation with Percoll (Kreamer et al., 1986). The cells were then preincubated in Krebs-Henseleit bicarbonate buffer (pH 7.4) supplemented with 12.5mM Hepes for 30 min in an atmosphere of 95% O_2/ 5% CO_2 or 1% O_2/ 5% CO_2/ 94% N_2 in continuously rotating 50mL round bottom flasks at 37^{oC} before the addition of chemicals (Figure 2.1). Stock solutions of chemicals were made either in incubation buffer or in methanol (maximum 0.1% with no significant effect on cell viability and the assays) and added to hepatocyte suspensions along with copper or iron complexes. GSH-depleted hepatocytes were obtained by 20 min preincubation of normal hepatocytes with

200μM 1-bromoheptane, which resulted in depletion of 95% of total hepatocyte GSH without affecting viability, as described previously (Khan and O'Brien, 1991). With this method, hepatocyte cytosolic and mitochondrial GSH can be extensively depleted as a result of a GSH transferase catalyzed GSH conjugation without causing cytotoxicity even at concentrations as high as 4mM 1-bromoheptane.

2.3. Cell Viability

The viability of the isolated hepatocytes was assessed by plasma membrane disruption determined by the trypan blue (0.2% w/v) exclusion test (Moldeus et al., 1978). Viability was examined at different time points during the 3 hour incubation and at least 80-90% of control cells were viable after 3 hours.

2.4. Lipid peroxidation

Hepatocyte lipid peroxidation was determined by measuring the amount of thiobarbituric acid-reactive substances (TBARS) formed during the decomposition of lipid hydroperoxides by following the absorbance at 532nm in a Beckman DU®-7 spectrophotometer after treating 1.0mL aliquots of hepatocyte suspension ($1x10^6$ cells/mL) with trichloroacetic acid (70%w/v) and boiling the supernatant with thiobarbituric acid (0.8% w/v) for 20 min (Smith et al., 1982).

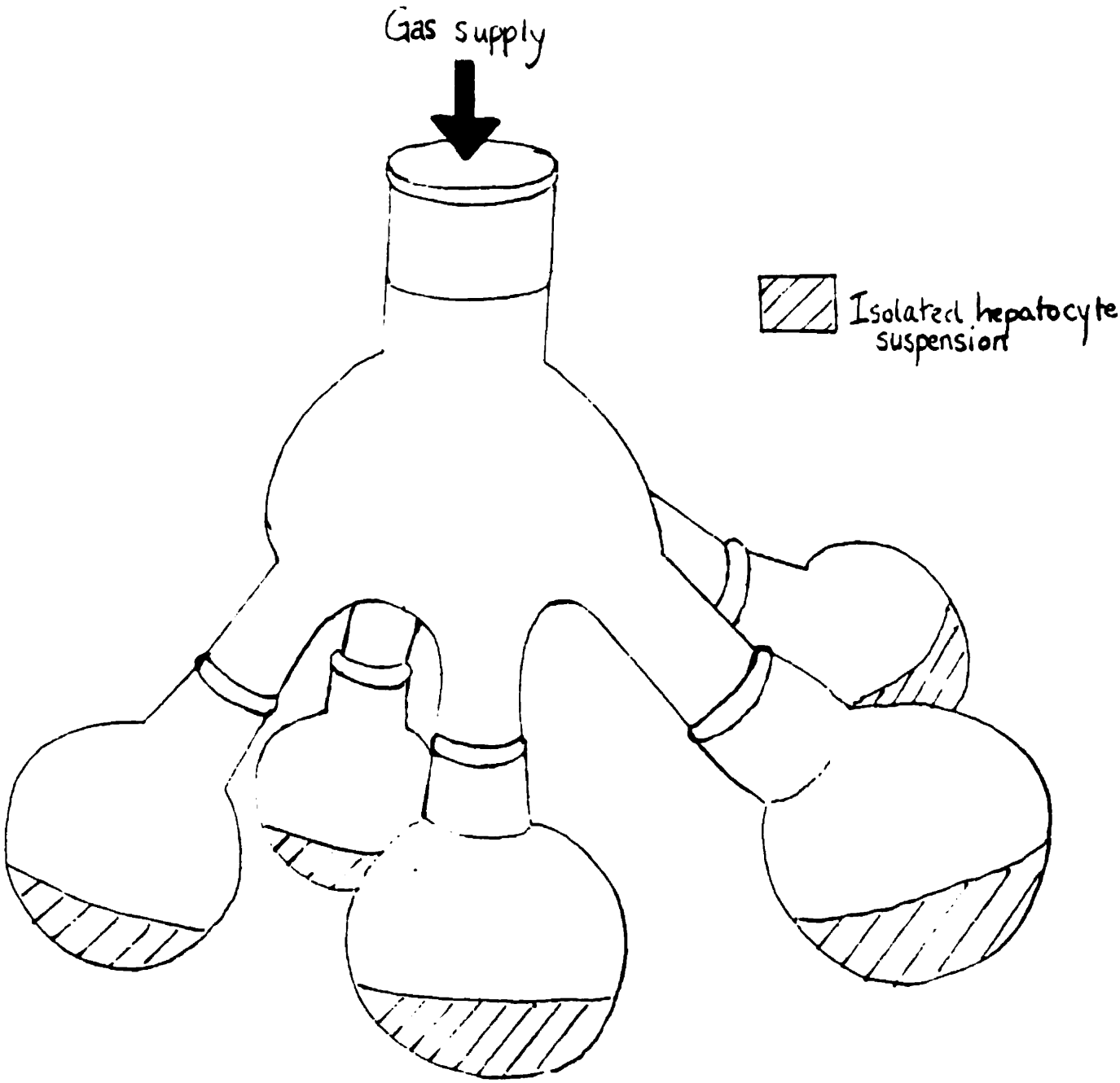


Figure 2.1: Diagram of the instrument used in hepatocyte isolation and incubation

2.5. Antimycin-A Resistant Respiration

Hepatocyte mitochondrial respiration was inhibited by the addition of antimycin A (25μM) and the rate of oxygen uptake was determined with a Clarke-type oxygen electrode (Model 5300; Yellow-Spring Instrument Co., Inc., Yellow Springs, OH, USA) following the addition of iron or copper complexes to hepatocytes in a 2-mL incubation chamber regulated at 37°C. These metal complexes were added after the baseline of the antimycin A-resistant respiration rate had stabilized.

2.6. HPLC Analysis of Hepatocyte GSH/GSSG

The total amount of hepatocyte GSH and GSSG were measured by the HPLC analysis of deproteinized samples (25% meta-phosphoric acid) after derivatization with iodoacetic acid and fluoro-2,4-dinitrobenzene (Reed et al., 1980) using a Waters HPLC system (Model 510 pumps, WISP 710B auto injector, and model 410 UV/VIS detector) equipped with a Waters μ Bondapak ® NH_2 (10μm) 3.9 x 300 mm column. Figure 2.2a and b are representative chromatograms of standards (GSH and GSSG) and sample.

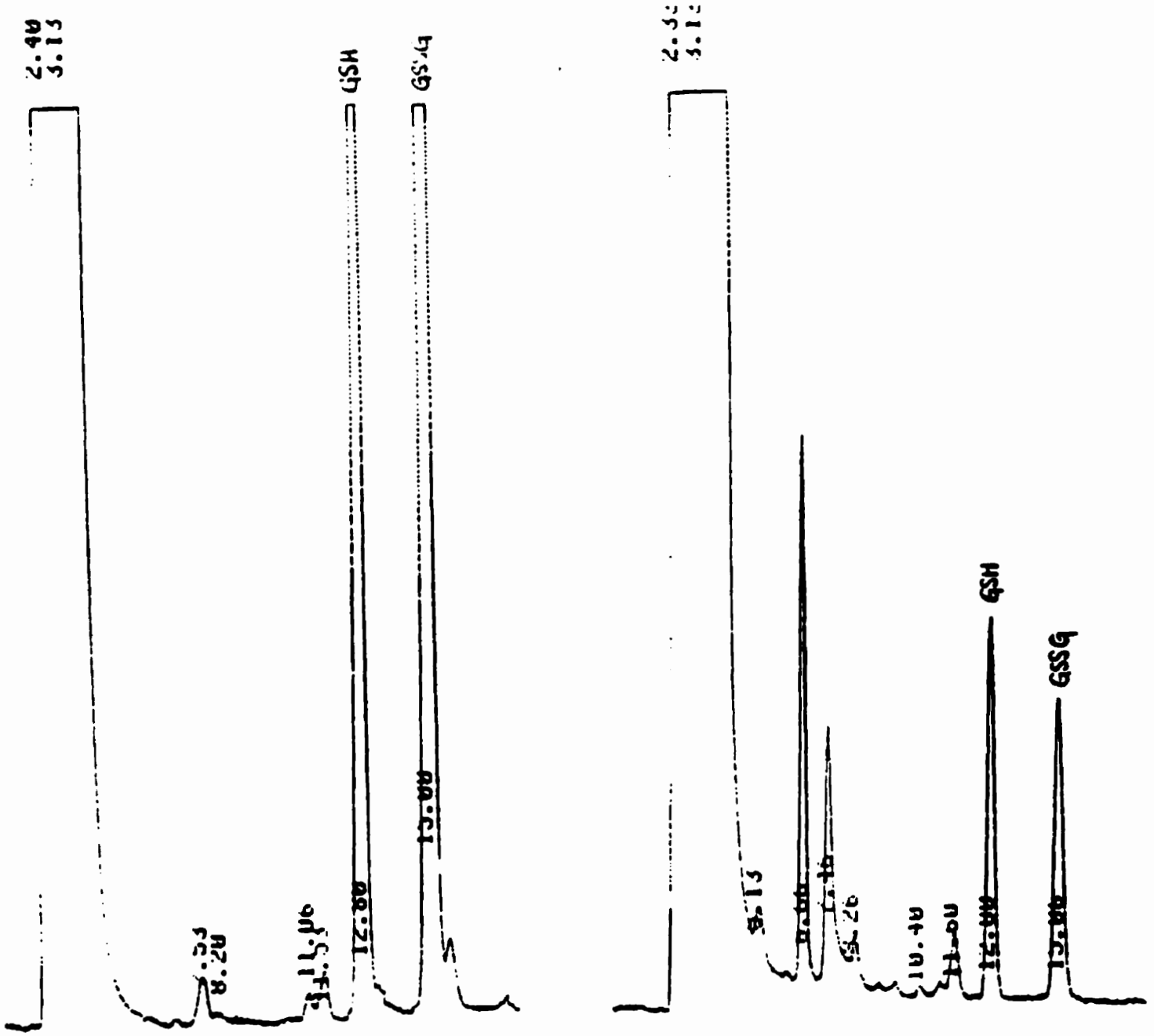


Fig 2.2a and b: Representation chromatograms of a) GSH and GSSG standards and b) hepatocyte sample

2.7. Determination of Hepatocyte Free Protein Sulfhydryl Groups

Aliquots of hepatocyte suspension (0.5mL) were obtained from respective incubation flasks and deproteinized in trichloroacetic acid (5% with 5mM EDTA). Samples were placed on ice and centrifuged for 10 min at 100 K rpm. The cell debris pellet was then resuspended in buffer (0.1mM Tris HCl, 5mM EDTA, 0.5% SDS w/v) and the 1-mL suspension was further diluted with Tris HCl buffer (pH 8.9). Freshly prepared DTNB (10mM) was added to complete the reaction and the absorbance at λ=412nm was measured with a Beckman DU®-7 spectrophotometer.

2.8. Determination of hepatocyte mitochondrial function

Hepatocyte mitochondria function was estimated from the uptake of the cationic fluorescent dye, rhodamine 123 (Aw and Jones, 1985). Aliquots (0.5mL) of the cell suspension were separated from the incubation medium by 1 min centrifugation at 1,000 rpm. Following this, cells were resuspended in fresh incubation medium (Krebs-Henseleit buffer, pH 7.4) containing Rhodamine 123 dye (1.5µM). Samples were then incubated in water bath at 37°C for 10 min and spectrofluorometrically analyzed using a Shimadzu RF5000U spectrofluorometer set at 490nm (excitation) and 525nm (emission). Results were expressed as % difference in fluorescence of total rhodamine 123 in sample aliquot compared to blank rhodamine 123, which reflects the capacity of mitochondria to take up cationic dye before and after incubation with metal complexes.

2.9. Determination of hepatocyte rate of reduction of copper complexes

The rates of reduction of copper complexes when added to isolated hepatocytes were followed with an Aminco DW2000 spectrophotometer set at the respective wavelengths of each cuprous complex. Hepatocytes (1x 10^6 cells/mL) were added to the sample cuvette before addition of the cupric complex. The cell mixture was consistently stirred with an automated magnetic stirrer at a fixed speed. Spectral acquisition was obtained over time to determine the relative rates of reduction for $Cu^{2+}(OP)_2$ and $Cu^{2+}(NC)_2$ by hepatocytes.

2.10. *In vivo* treatment of animals and preparation for enzyme assays

CD1 mice, 25-30g, were obtained from Charles River Canada Laboratories (Montreal, PQ.), fed *ad libitum*, and were allowed to acclimatize for at least 1 week on clay chip bedding. All mice were injected intraperitoneally with $Cu^{2+}(NTA)_2$ (0.23mmol/kg) and treated where indicated with desferoxamine (35-40mg/kg), or DMSO (4-6mL/kg), or leupeptin (0.5mg/kg) or GSH (5mmol/kg). Control mice were injected with deionized water. All mice were sacrificed 24 hours later by heart puncture.

Serum extracted from heart puncture was placed in Eppendorf centrifuge tubes on ice and centrifuged at 1060 rpm for 10 min in an ultracentrifuge. Clear supernatant was decanted carefully and kept at 0°C for enzyme assays.

2.11. Blood Urea Nitrogen (BUN) levels from treated mice

A Sigma Diagnostic Kit for the BUN assay was used to ascertain the extent of kidney damage induced by $Cu^{2+}(NTA)_2$. Serum samples (10μL) were added to 0.5mL urease solution and incubated at 37°C for 10-15 min. Another 15-20 min was allowed to enable the urease to hydrolyze serum urea to ammonia at room temperature. The absorbance at λ=570nm of samples was analysed on the spectrophotometer upon development of the color with addition of the reagents in the order of phenol nitroprusside (catalyst) and alkaline hypochlorite. The levels of Urea Nitrogen (mg/dL) in the sample were then determined from the calibration curve.

2.12. Serum transaminases (ALT and AST) from treated mice

Increased serum transaminase levels are indicative of liver damage. The assay for these enzymes was performed with the respective Sigma Diagnostic Kit. For the ALT assay, the substrates used were alanine-(α-KG) whereas the AST assay required the substrates, aspartate-(α-KG). The color formation requires 2,4-dinitrophenol (DNP) and is spectrophotometrically analyzed at 505nm. The respective substrates are reacted with sample serum (20μL) and incubated for 30-60 min. The absorbance at 505nm was measured after the transamination reaction was inhibited by addition of 2,4-dinitrophenol solution and 0.40N NaOH. The ALT and AST activities (SF Units/mL) were then estimated from the calibration curves.

2.13. Preparation of ferric and cupric nitrilotriacetate

A 10 mM ferric nitrilotriacetate (1:2) solution was prepared prior to use by dissolving 47 mg nitrilotriacetate with 200mg ferric ammonium citrate in 30 mL distilled H_2O (Morel et al., 1990). The pH was adjusted to 7.0 with $NaHCO_3$.

A 10 mM cupric nitrilotriacetate (1:2) solution was freshly prepared from 25mg cupric sulfate, 60mg of nitrilotriacetate in 1.0 mL distilled H_2O (Toyokuni et al., 1989).

2.14. Statistical Analysis

One and two-way ANOVA followed by the Scheffe's test were used for comparison amongst the multiple-treated groups and the relevant controls. Results represent the mean ± standard error of the mean (SEM) of triplicate samples.

Chapter 3

CONTRASTING THE CYTOTOXIC MECHANISMS OF COPPER AND IRON WITH ISOLATED HEPATOCYTES *IN VITRO* AND *IN VIVO*

3.1. INTRODUCTION

Both iron and copper are essential metals that are abundant in many proteins and often participate in biological and biochemical catalytic pathways. Iron forms the active centers of a variety of enzymes, namely, ribonucleotide reductase, aconitase, cytochromes and other heme proteins. Copper is essential in metalloenzymes such as ceruloplasmin, superoxide dismutase, lysyl oxidase and cytochrome c oxidase (Silva and Aust, 1992). Evolution has provided the cell physiological stores for the careful regulation of these trace metals, yet upon their leakage out of stores, these "free" unbound metals become deleterious in affected tissues (Aust et al., 1985; Halliwell and Gutteridge, 1988; Edwards et al., 1991; Lesnefsky, 1992). These trace metals have been implicated in the role of free radical reactions, which lead to oxidative cellular damage either from DNA base modification (Iqbal et al., 1995; Toyokuni et al., 1995), mitochondrial damage (Carini et al., 1992; Korge and Campbell, 1993; Itoh et al., 1994) or lysosomal membrane damage (Myers et al., 1993; Ollinger and Brunk, 1995).

Iron and copper overload are pathological situations where plasma concentrations of iron and copper exceed several-fold the total binding capacity of transferrin and ceruloplasmin or transcuprein, respectively. Clinical consequences of iron overload

include hereditary haemochromatosis, which involves abnormally high gut absorption of iron with unparalleled presence of plasma circulating transferrin; β-thalassemia, Parkinson's and Hallervorden Spatz disease, all of which have elevated levels of iron in the liver or brain respectively (Youdim et al., 1993).

Wilson's disease is an example of copper overload, resulting from a genetic disorder in the expression of the copper-dependent ATPase transporter (Tanzi et al., 1993; Bull et al., 1993). Other examples of copper overload include toxic-milk mice that results from a recessive disorder of copper metabolism in female mother mouse (Rauch et al., 1986) and the phenotypical Indian childhood cirrhosis (ICC) that are related to both environmental and genetic factors (Alt et al., 1990; Danks, 1991; Joshi, 1987). In all cases of copper overload, there is generally excessive copper accumulation in the liver that eventually leads to liver damage. In Wilson's disease, other pathological consequences include renal tubular dysfunction, pigmented corneal rings (Kayser-Fleischer rings) and the late onset of neurologic disorders (Sternlieb, 1990).

In recent investigations, animal iron and copper overload models have been developed using iron nitrilotriacetate and copper nitrilotriacetate so as to understand the clinical consequences of iron or copper overload respectively (Sugawara et al., 1991; Dabbagh et al., 1994). Nitrilotriacetic acid (NTA) is a synthetic aminopolycarboxylic acid that chelates metals to form water-soluble complexes at neutral pH (Goddard and Sweeney, 1983). It has various household and industrial uses as a detergent. Due to its

commercial use, it has been the subject of numerous investigations. It was discovered that animals treated with Fe^{3+} $(NTA)_2$ experienced tissue iron overloading and the animals developed renal adenocarcinoma and proximal tubular necrosis (Li et al., 1987; Yamanoi et al., 1982). Dabbagh et al. (1994) demonstrated in Sprague-Dawley male rats that iron overloading led to a significant decrease in the concentrations of plasma antioxidants, namely α-tocopherol and ascorbic acid and liver antioxidants, including α-tocopherol, β-carotene and ubiquinol-10. *In vitro* investigations were also carried out in hepatocytes by Carini et al. (1992). Iron overloading of isolated hepatocytes with $Fe^{3+}(NTA)_2$ showed mitochondrial membrane damage, a loss in mitochondrial membrane potential and a loss of ATP. Itoh et al. (1994) demonstrated that iron ion induced mitochondrial DNA damage in HTC rat hepatoma cell cultures was prevented by α-tocopherol and Coenzyme Q homologs.

Toyokuni et al. (1989) reported that rats treated with daily doses of Cu^{2+} $(NTA)_2$ suffered from central to submassive liver necrosis, which included extensive fibrosis and cirrhosis. It is also well known that hemolytic anemia is associated with acute toxicity of copper overload. Hochstein et al. (1978) investigated this effect of copper on red cell membrane and suggested that copper initiates the formation of hydrogen peroxide and peroxidation of lipids via conjugation with membrane sulfhydryl groups.

The objective of this study was to determine possible differences between the cytotoxic mechanisms of Fe^{3+} $(NTA)_2$ and Cu^{2+} $(NTA)_2$, even though both complexes

cause renal and hepatotoxicity and both Fe and Cu are redox-active metals which participate in oxygen activation reactions. In the following, profound differences were observed between their respective cytotoxic mechanisms as Fe^{3+} $(NTA)_2$ toxicity was completely protected against by antioxidants (DPPD and BHA) and Cu^{2+} $(NTA)_2$ toxicity was completely prevented by the thiol reductant (DTT) and ROS scavengers (SOD and catalase). Cytotoxicity and lipid peroxidation by Cu^{2+} $(NTA)_2$ was prevented by the ferric chelator, desferoxamine and the copper chelator, ammonium tetrathiomolybdate.

3.2. RESULTS

3.2.1. Effect of chelation on metal toxicity

Figure 3.1a represents the dose-dependence cytotoxicity of Cu^{2+} $(NTA)_2$ incubated with normal and GSH-depleted hepatocytes whereas Figure 3.1b represents the dose-dependence cytotoxicity of Fe^{3+} $(NTA)_2$. The concentration of Cu^{2+} $(NTA)_2$ required to cause 50% cytotoxicity in 2 hours was estimated to be 30μM whereas the concentration of NTA required was about 1mM. After 3 hour-incubations with isolated hepatocytes, Cu^{2+} $(NTA)_2$ (30μM) proved to be more toxic than Cu^{2+} (30μM) and caused 70% and 38% cytotoxicity respectively (Figure 3.2). The concentration of Fe^{3+} $(NTA)_2$ required to cause 50% cytotoxicity was 180μM whereas the concentration of Fe^{3+} required was 280μM.

3.2.2. Modulation of toxicity with antioxidants, reductants and scavengers

As shown in Figure 3.3, Cu^{2+} $(NTA)_2$ toxicity was prevented effectively by the •OH radical scavengers (DMSO and mannitol), or by ROS scavengers, catalase (20U) and SOD (0.5μg), but less effectively from antioxidants (DPPD and BHA). By contrast, Fe^{3+} $(NTA)_2$ toxicity was only slightly protected by the •OH radical scavengers (DMSO and mannitol) or catalase or SOD as shown in Figure 3.4. Antioxidants (DPPD and BHA) were effective in preventing $Fe^{3+}(NTA)_2$ toxicity.

In Figures 3.5 and 3.6 respectively, toxic Cu^{2+} NTA concentrations formed 3-4 fold less lipid peroxidation than Fe^{3+} $(NTA)_2$ despite Cu^{2+} $(NTA)_2$ being much more toxic. Indeed Fe^{3+} $(NTA)_2$ still induced lipid peroxidation at lower noncytotoxic concentrations (eg. 25μM). Lipid peroxidation induced by Cu^{2+} $(NTA)_2$ was decreased by desferoxamine, which suggested that iron contributes to Cu^{2+} $(NTA)_2$ -induced lipid peroxidation. Antioxidants, DPPD (1μM) and BHA (50μM) prevented lipid peroxidation induced by both chelates. However, whilst Fe^{3+} $(NTA)_2$ -induced cytotoxicity was prevented, Cu^{2+} $(NTA)_2$ -induced cytotoxicity was not affected. Furthermore DMSO, mannitol, and catalase, did not prevent lipid peroxidation induced by either Cu^{2+} $(NTA)_2$ or $Fe^{3+}(NTA)_2$.

3.2.3. Determination of free protein sulfhydryls and thiols

Protein thiols participate in the uptake and distribution of iron and copper intracellularly (Spear and Aust, 1994). In Figure 3.7, both Cu^{2+} $(NTA)_2$ and Fe^{3+} $(NTA)_2$ depleted protein sulfhydryl ligands relative to control isolated rat hepatocytes. Cu^{2+} $(NTA)_2$ was 6-fold more potent in depleting protein sulfhydryls than Fe^{3+} $(NTA)_2$.

As shown in Figure 3.8a and b, cytotoxic concentrations of Cu^{2+} $(NTA)_2$ were much more effective in depleting GSH levels and forming GSSG than Fe^{3+} $(NTA)_2$. In another experiment, GSH depleted hepatocytes were 6-fold more sensitive to Cu^{2+} $(NTA)_2$ and only 2-fold more sensitive to Fe^{3+} $(NTA)_2$ compared to control hepatocytes (Table 3.1) This suggests that GSH plays a crucial role in detoxifying intracellular "free"

Cu^{2+} / Fe^{3+} .

3.2.4. Effect of modulators on Cu^{2+} / Fe^{3+} induced loss of mitochondria function

As observed in Figure 3.9, cytotoxic concentrations of Cu^{2+} $(NTA)_2$ and Fe^{3+} $(NTA)_2$ caused a loss in mitochondria function. However, the reactive species responsible were different for Cu^{2+} $(NTA)_2$ and Fe^{3+} $(NTA)_2$. The mitochondrial toxicity induced by Cu^{2+} $(NTA)_2$ but not Fe^{3+} $(NTA)_2$ was prevented significantly by catalase (20U) or DMSO. However, the antioxidant, DPPD (1μM) prevented mitochondrial toxicity induced by Fe^{3+} $(NTA)_2$ but not Cu^{2+} $(NTA)_2$.

3.2.5. Increased hepatocyte susceptibility at physiological oxygen concentrations

Hepatocytes were more susceptible to both Cu^{2+} $(NTA)_2$ and Fe^{3+} $(NTA)_2$ at 1% O_2/ 5% CO_2/ 94% N_2 than at 95% O_2/ 5% CO_2 suggesting that mitochondrial toxicity contributed to hepatocyte cytotoxicity (Figure 3.10a). Cytotoxicity was also prevented by glutamine (2mM), an ATP generator. Ruthenium red (50μM) which inhibits mitochondrial Ca^{2+} recycling also prevented cytotoxicity (Figure 3.10b).

3.2.6. Possible therapy against hepatocyte Cu^{2+} $(NTA)_2$ and $Fe^{3+}(NTA)_2$ toxicity

As shown in Figure 3.11 , Cu^{2+} $(NTA)_2$ toxicity was prevented by the copper

chelator, ammonium tetrathiomolybdate (100μM), D-penicillamine (1mM) and the ferric chelator, desferoxamine (500μM). Wilson's disease is currently treated with D-penicillamine and tetrathiomolybdate is currently in clinical trials. Liver iron overload is treated with desferoxamine. The lysosomal cysteine proteinase inhibitor, E64 (20μg) and the thiol reductant, DTT also prevented Cu^{2+} $(NTA)_2$ toxicity.

As shown in Figure 3.12, Fe^{3+} $(NTA)_2$ toxicity was also prevented by the ferric chelator, desferoxamine. However, the copper chelators, ammonium tetrathiomolybdate and D-penicillamine, or the thiol reductant, DTT had no effect.

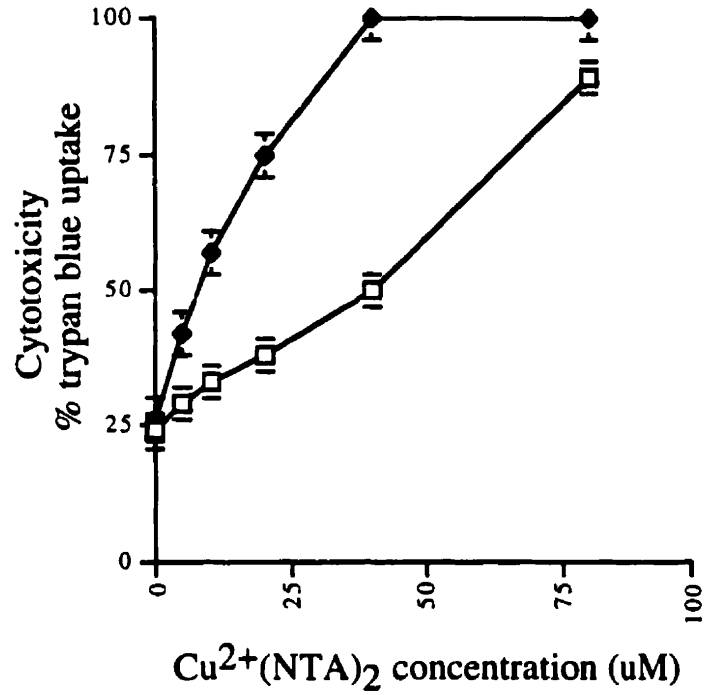


Figure 3.1a: Dose dependence cytotoxicity of $Cu^{2+}(NTA)_2$ determined with normal (❑) and GSH depleted hepatocytes (◆).

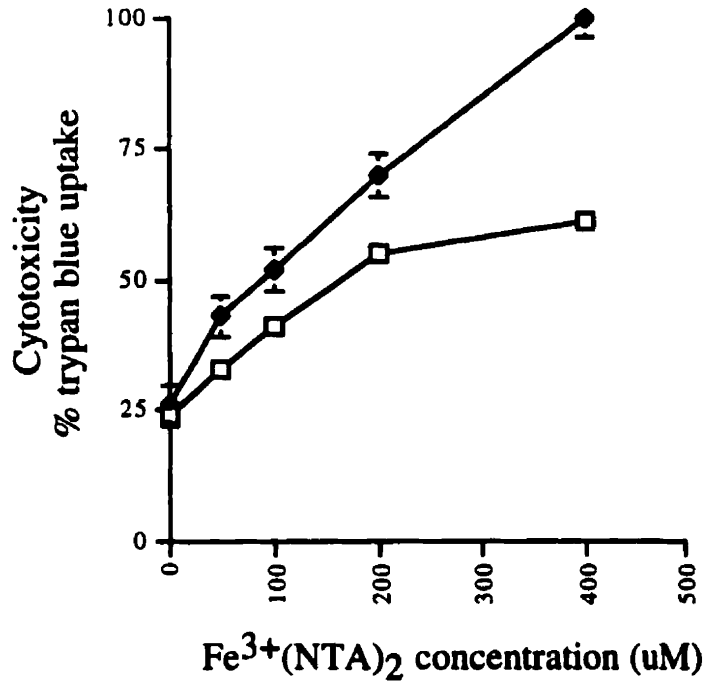


Figure 3.1b: Dose dependence cytotoxicity of $Fe^{3+}(NTA)_2$ determined with normal (❑) and GSH depleted hepatocytes (◆).

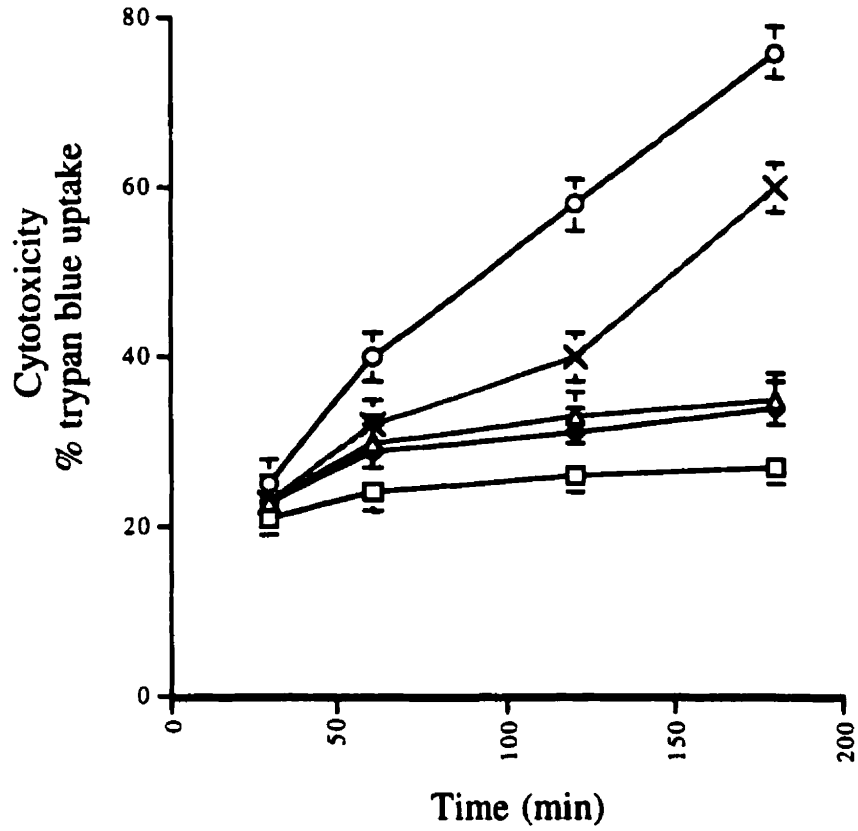


Figure 3.2: Effect of NTA chelation on copper and iron toxicity towards isolated hepatocytes. Hepatocytes (1 x 10^6 cells/mL) were incubated with Cu^{2+}(30μM) (Δ), Cu^{2+} $(NTA)_2$ (30μM) (O), Fe^{3+} (180μM) (◆) and Fe^{3+} $(NTA)_2$ (180μM) (X). Control hepatocytes (❑) with no additions. It was observed that Cu^{2+} $(NTA)_2$ and Fe^{3+} $(NTA)_2$ were significantly more toxic than the "free" metals and Cu^{2+} $(NTA)_2$ was approximately 6-fold more toxic than Fe^{3+} $(NTA)_2$. Values represent mean ± SE of three separate experiments.

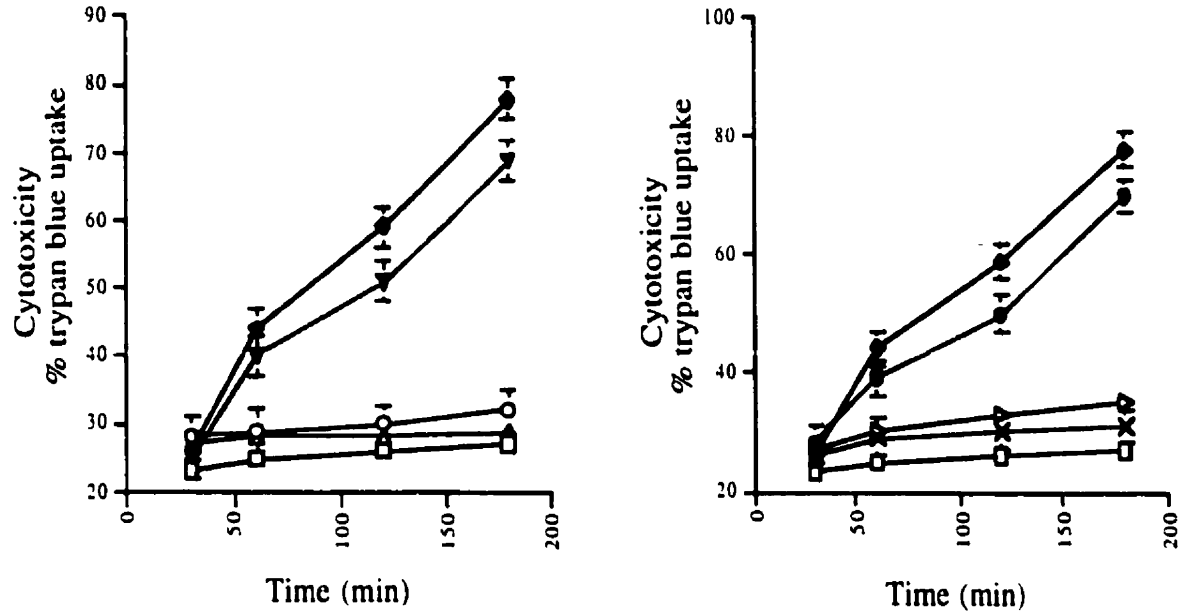


Figure 3.3 : Cu^{2+} $(NTA)_2$ -induced hepatocyte cytotoxicity is prevented by ROS scavengers but not antioxidants. Hepatocytes (1 x 10^6 cells/mL) were incubated with Cu^{2+} $(NTA)_2$ (30μM) (◆). **(Left):** Cu^{2+} $(NTA)_2$ + DMSO (100μL) (Δ), Cu^{2+} $(NTA)_2$ + SOD (0.5μg) (○), and Cu^{2+} $(NTA)_2$ + DPPD (1μM) (▼). **(Right):** Cu^{2+} $(NTA)_2$ + mannitol (30mM) (X), Cu^{2+} $(NTA)_2$ + catalase (20U) (◗), and Cu^{2+} $(NTA)_2$ + BHA (50μM) (●). Control hepatocytes (❑) with no additions. Values represent mean ± SE of three separate experiments.

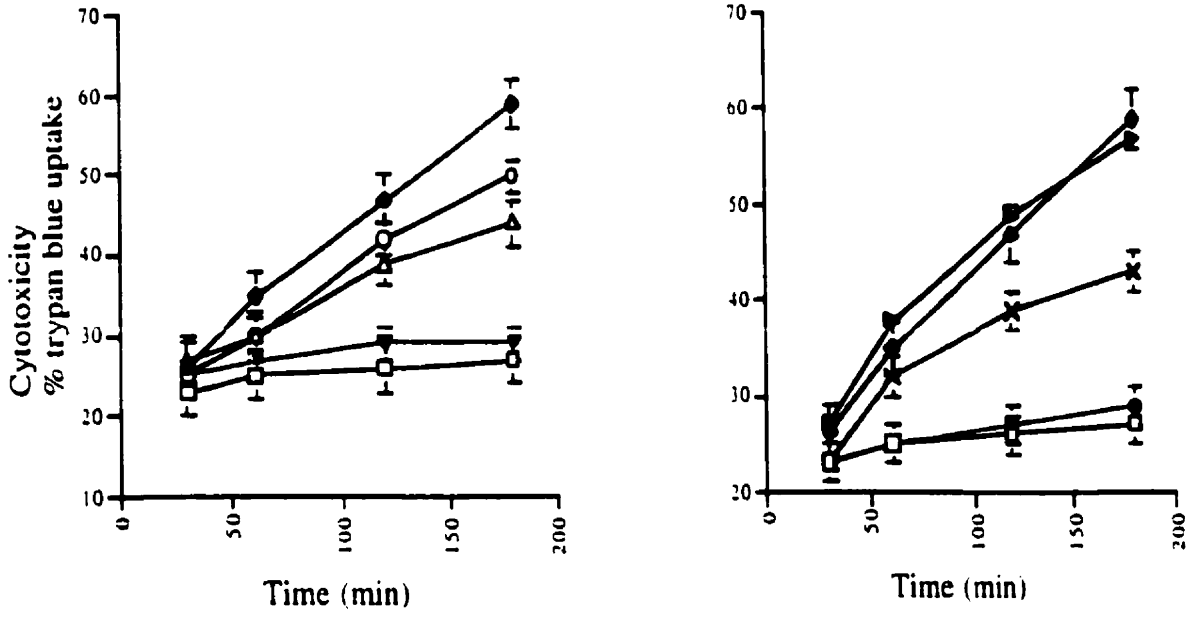


Figure 3.4 : Fe^{3+} $(NTA)_2$ -induced hepatocyte cytotoxicity is prevented by antioxidants but not by ROS scavengers. Hepatocytes (1 x 10^6 cells/mL) were incubated with Fe^{3+} $(NTA)_2$ (180μM) (◆). **(Left):** Fe^{3+} $(NTA)_2$ + DMSO (100μL) (Δ), Fe^{3+} $(NTA)_2$ + SOD (0.5μg) (○), and Fe^{3+} $(NTA)_2$ + DPPD (1μM) (▼). **(Right):** Fe^{3+} $(NTA)_2$ + mannitol (30mM) (X), Fe^{3+} $(NTA)_2$ + catalase (20U) (◗), and Fe^{3+} $(NTA)_2$ + BHA (50μM) (●). Control hepatocytes (❑) with no additions. Values represent mean ± SE of three separate experiments.

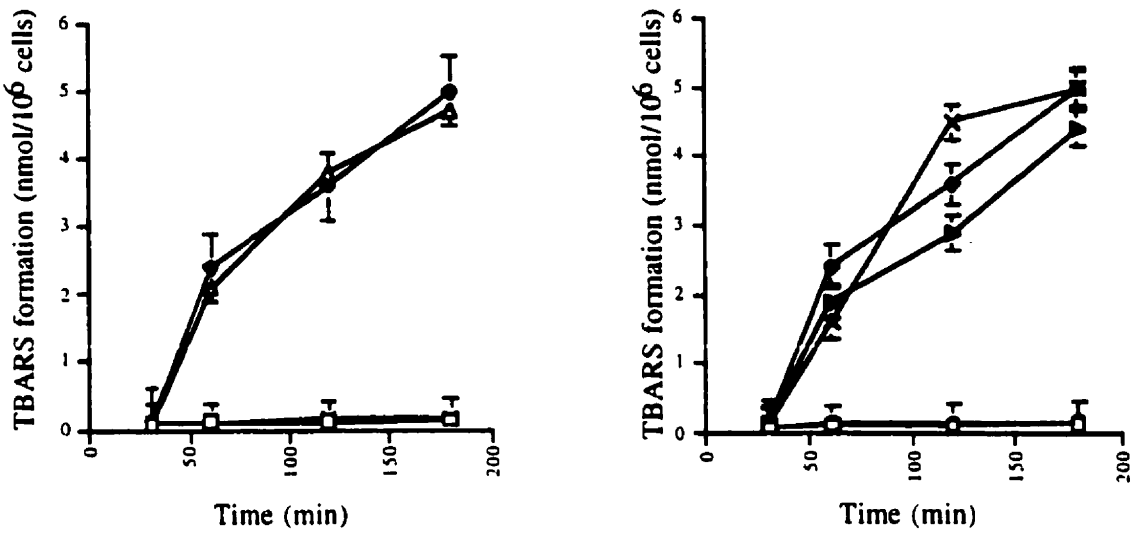


Figure 3.5: Cu^{2+} $(NTA)_2$ -induced hepatocyte lipid peroxidation is prevented by antioxidants, iron chelator but not by ROS scavengers. Hepatocytes (1 x 10^6 cells/mL) were incubated with Cu^{2+} $(NTA)_2$ (30μM) (◆). **(Left):** Cu^{2+} $(NTA)_2$ + DMSO (100μL) (Δ),Cu^{2+} $(NTA)_2$ + desferoxamine (100μM) (○), and Cu^{2+} $(NTA)_2$ + DPPD (1μM) (▼) **(Right):** Cu^{2+} $(NTA)_2$ + mannitol (30mM) (X), Cu^{2+} $(NTA)_2$ + catalase (20U) (◗), and Cu^{2+} $(NTA)_2$ + BHA (50μM) (●). Cu^{2+} $(NTA)_2$ lipid peroxidation was prevented by antioxidants and desferoxamine. Control hepatocytes (❑) with no additions. Values represent mean ± SE of three separate experiments.

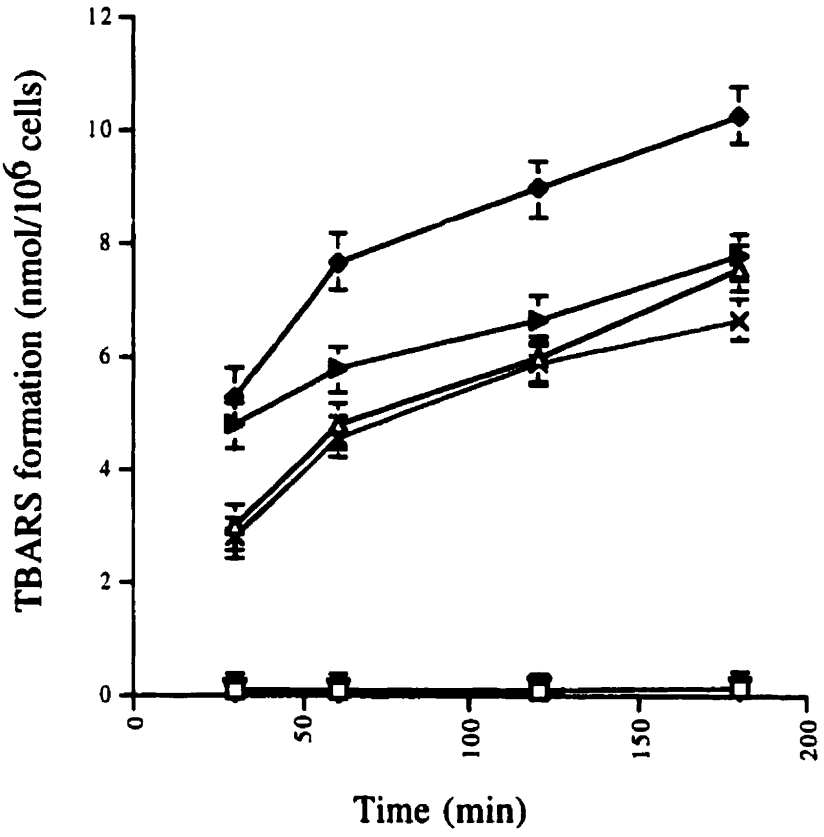


Figure 3.6: Fe^{3+} $(NTA)_2$ -induced lipid peroxidation is prevented by antioxidants and iron chelator but not by ROS scavengers. Hepatocytes (1 x 10^6 cells/mL) were incubated with Fe^{3+} $(NTA)_2$ (180μM) (◆), Fe^{3+} $(NTA)_2$ + DMSO (100μL) (Δ), Fe^{3+} $(NTA)_2$ + mannitol (30mM) (X), Fe^{3+} $(NTA)_2$ + desferoxamine (100μM) (○), Fe^{3+} $(NTA)_2$ + catalase (20U) (◗), Fe^{3+} $(NTA)_2$ + DPPD (1μM) (▼) and Fe^{3+} $(NTA)_2$ + BHA (50μM) (●). Control hepatocytes (❑) with no additions. Values represent mean ± SE of three separate experiments.

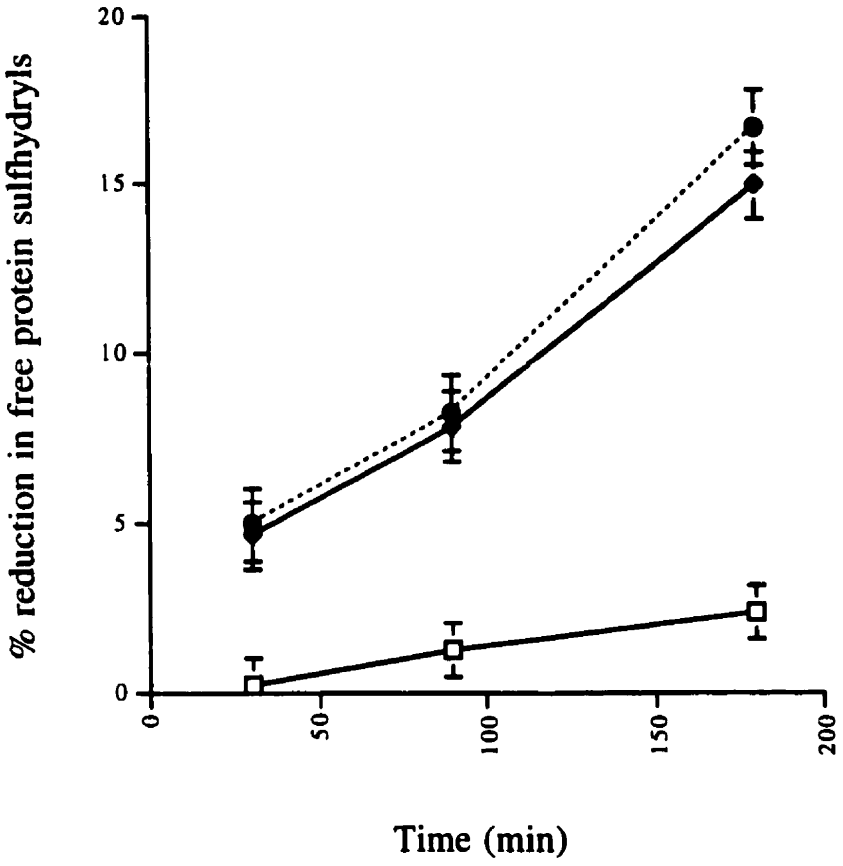
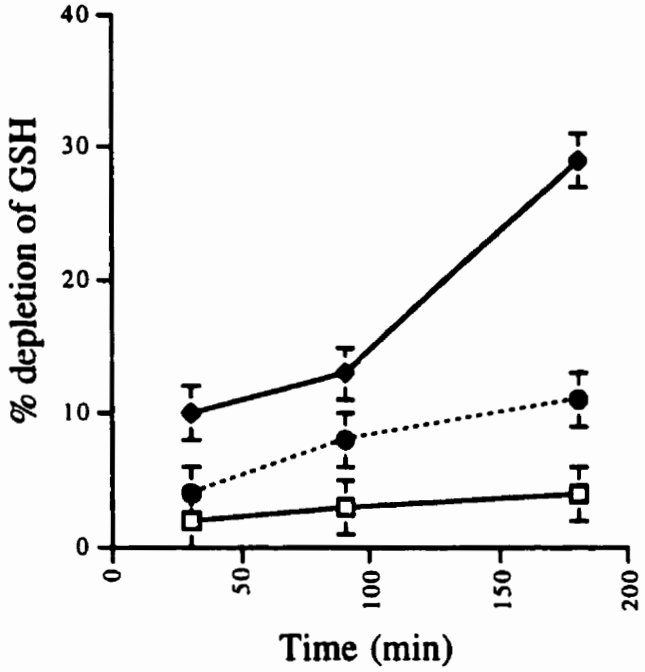


Figure 3.7: Depletion of protein thiols in isolated hepatocytes incubated with Cu^{2+} $(NTA)_2$ (30μM) (◆) and Fe^{3+} $(NTA)_2$ (180μM) (●). Both metal complexes caused significant depletion in free protein thiols compared to control hepatocytes (❑) with no additions. Values represent mean ± SE of three separate experiments.



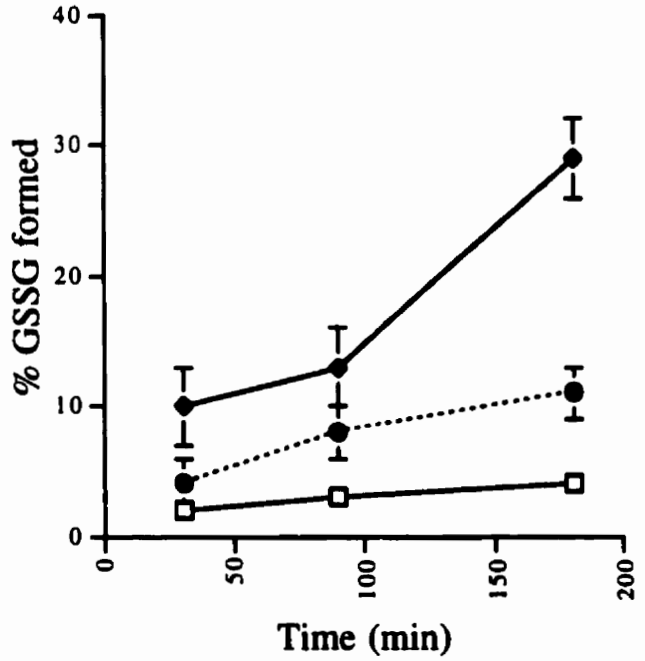


Figure 3.8a and b: Hepatocyte depletion of reduced GSH and GSSG formation induced by 30μM Cu^{2+} $(NTA)_2$ (◆) and 180μM Fe^{3+} $(NTA)_2$ (●) relative to control hepatocytes (❑) with no additions. Values represent mean ± SE of three separate experiments.

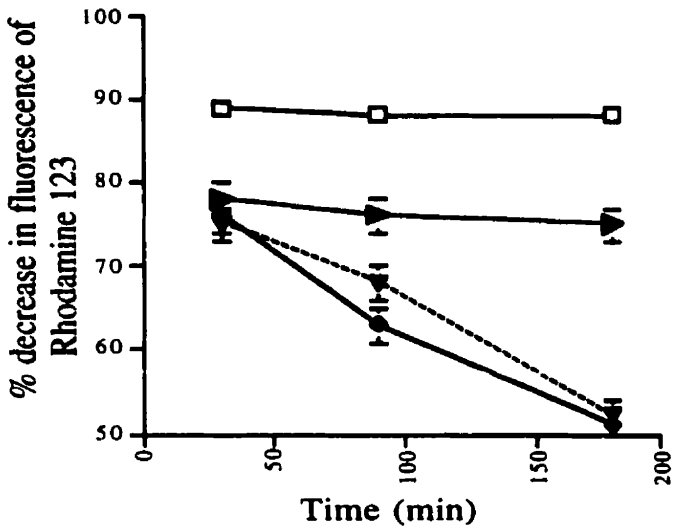


Figure 3.9a: Cu^{2+} $(NTA)_2$ -induced loss of mitochondria function in isolated rat hepatocytes. Hepatocytes (1 x 10^6 cells/mL) were incubated with Cu^{2+} $(NTA)_2$ (30μM) (◆), Cu^{2+} $(NTA)_2$ + catalase (20U) (◗) and Cu^{2+} $(NTA)_2$ + DPPD (1μM) (▼). Control hepatocytes (❑) had no additions. Loss in mitochondrial function was prevented by catalase and not DPPD. Values represent mean ± SE of three separate experiments.

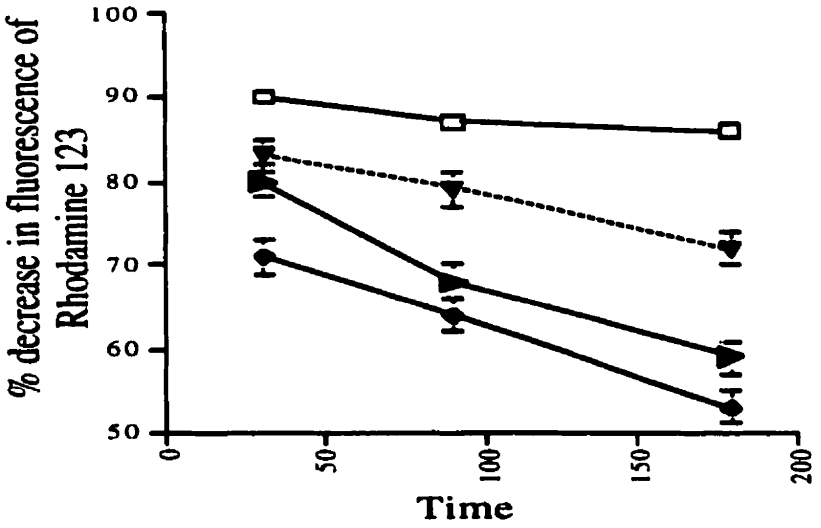


Figure 3.9b: Fe^{3+} $(NTA)_2$ -induced loss of mitochondrial function in isolated rat hepatocytes. Hepatocytes (1 x 10^6 cells/mL) were incubated with Fe^{3+} $(NTA)_2$ (180μM) (◆), Fe^{3+} $(NTA)_2$ + catalase (20U) (◗) and Fe^{3+} $(NTA)_2$ + DPPD (1μM) (▼). Control hepatocytes (❑) had no additions. Loss in mitochondrial function induced by Fe^{3+} $(NTA)_2$ was prevented by DPPD and not catalase. Values represent mean ± SE of three separate experiments.

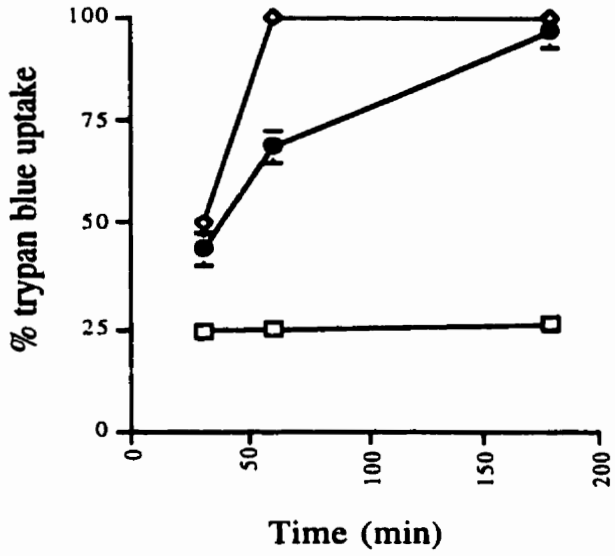


Figure 3.10a : Increased senstivity of isolated hepatocytes at 1% O_2 to both Cu^{2+} $(NTA)_2$ and Fe^{3+} $(NTA)_2$. Hepatocytes (1 x 10^6 cells/mL) were incubated with Cu^{2+} $(NTA)_2$ (30μM) (◆) and Fe^{3+} $(NTA)_2$ (180μM) (●). Control hepatocytes (❑) with no additions. Values represent mean ± SE of three separate experiments.

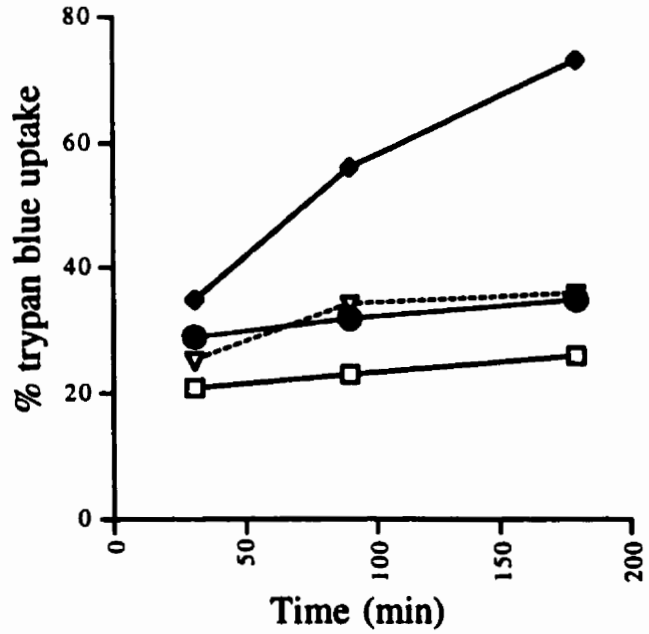


Figure 3.10b : Ruthenium red or glutamine prevents Cu^{2+} $(NTA)_2$ induced hepatocyte cytotoxicity . Hepatocytes (1 x 10^6 cells/mL) were incubated with Cu^{2+} $(NTA)_2$ (30μM) (◆), Cu^{2+} $(NTA)_2$ + ruthenium red (50μM) (Δ) and Cu^{2+} $(NTA)_2$ + glutamine (2mM) (●). Control hepatocytes (❑) with no additions.

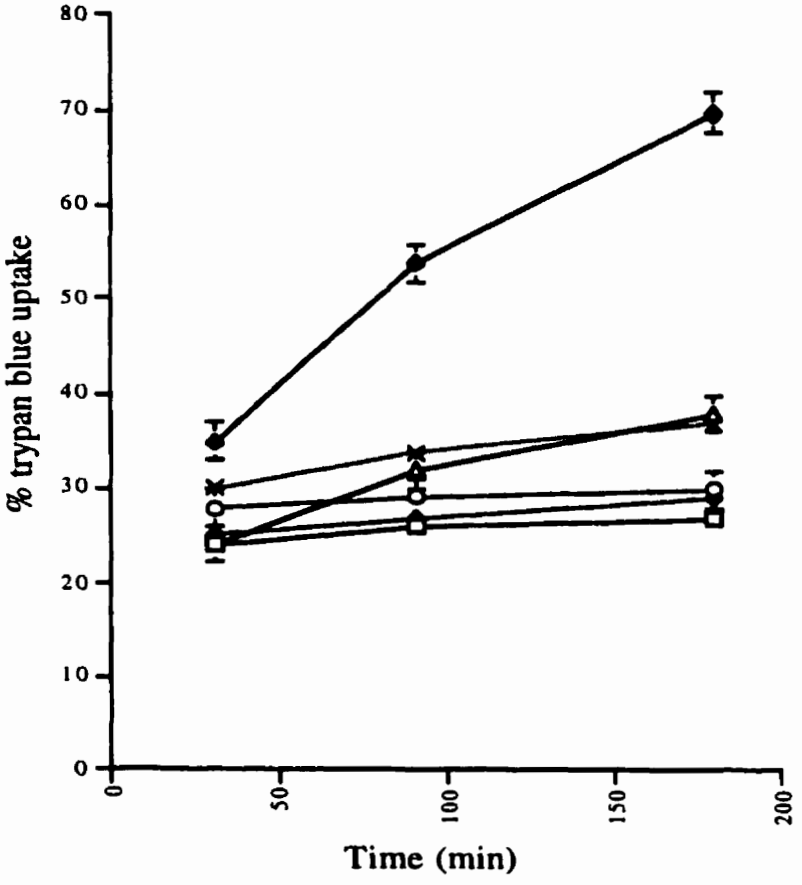


Figure 3.11: Prevention of Cu^{2+} $(NTA)_2$ -induced hepatotoxicity *in vitro* by copper chelators, ammonium tetrathiomolybdate and D-penicillamine; ferric chelator, desferoxamine; thiol reductant, DTT and lysosomal cysteine proteinase inhibitor, E64. Hepatocytes (1 x 10^6 cells/mL) were incubated with Cu^{2+} $(NTA)_2$ (30μM) (◆), Cu^{2+} $(NTA)_2$ + desferoxamine (100μM) (+), Cu^{2+} $(NTA)_2$ + D-penicillamine (1mM) (X), Cu^{2+} $(NTA)_2$ + TTM (100μM) (○), Cu^{2+} $(NTA)_2$ + DTT (3mM) (◗) and Cu^{2+} $(NTA)_2$ + E64 (20μg) (Δ). Control hepatocytes (❑) with no additions. Values represent mean ± SE of three separate experiments.

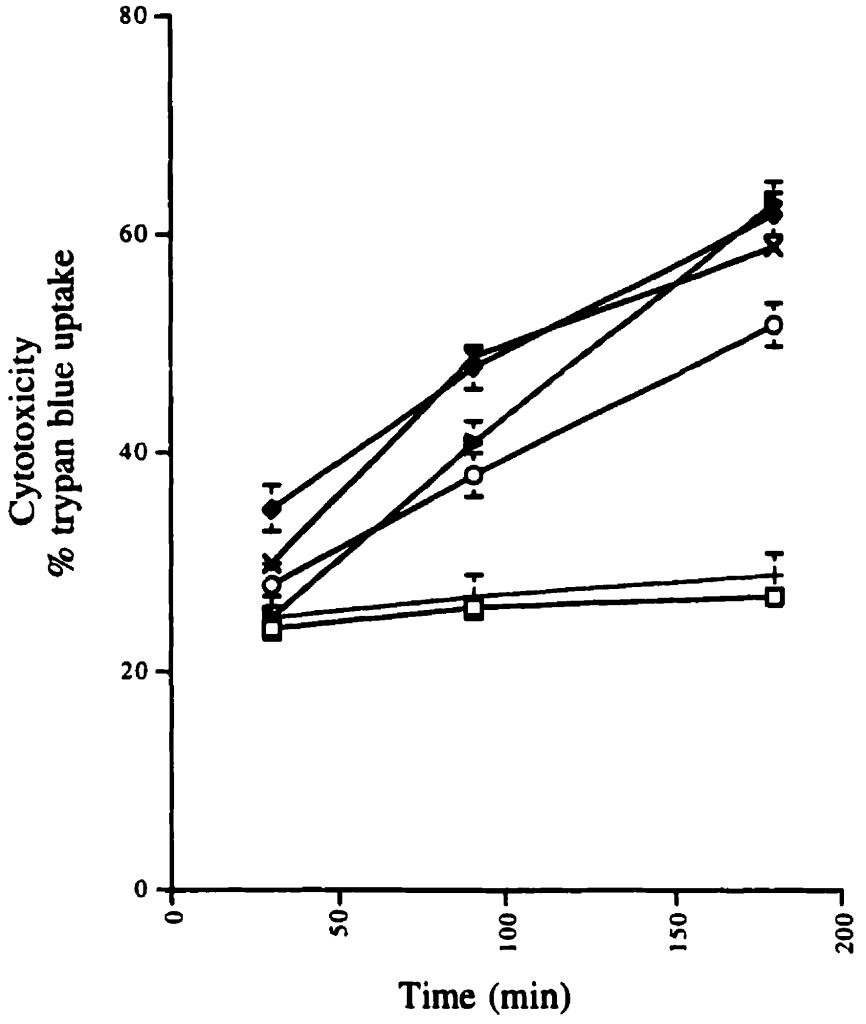


Figure 3.12: Modulation of Fe^{3+} $(NTA)_2$ -induced hepatotoxicity *in vitro* by ferric chelator, desferoxamine; thiol reductant, DTT and copper chelators, ammonium tetrathiomolybdate and L-penicillamine. Hepatocytes (1 x 10^6 cells/mL) were incubated in Fe^{3+} $(NTA)_2$ (180μM) (◆) , Fe^{3+} $(NTA)_2$ + desferoxamine (100μM) (+), Fe^{3+} $(NTA)_2$ + D-penicillamine (100μM) (X) , Fe^{3+} $(NTA)_2$ + TTM (100μM) (O) and Fe^{3+} $(NTA)_2$ + DTT (3mM) (◗). Control hepatocytes (❑) with no additions. Values represent mean ± SE of three separate experiments.

3.2.7. *In vivo* $Cu^{2+}(NTA)_2$ treatment of CD1 mice

Hepatotoxicity was determined in CD1 mice (25-30g) 24 hours after an intraperitoneal injection of $Cu^{2+}(NTA)_2$ (0.17-0.34 mmol/kg) by measuring plasma levels of ALT and AST (serum transaminases). Hepatotoxicity was dose-dependent (Figure 3.13a) and the desired $Cu^{2+}(NTA)_2$ dose used for modulating the *in vivo* treatment in CD1 mice was 0.23 mmol/kg. As shown in Figure 3.14a and 3.14b, hepatotoxicity was completely prevented by the administration of the copper antagonist, ammonium tetrathiomolybdate (10mg/kg), the ferric chelator, desferoxamine (35mg/kg) and the lysosomal protease inhibitor, leupeptin (1.0mg/kg). The •OH radical scavenger, DMSO (4mL/kg) failed to protect. By contrast, excess GSH (5mmol/kg) failed to prevent liver damage, possibly as a result of the reductive activation of $Cu^{2+}(NTA)_2$ by cysteine. GSH is rapidly hydrolysed to cysteine *in vivo* by the action of kidney γ-GTP and dipeptidase. Each treatment group consisted of 2-3 CD1 male mice. Experiments were performed in triplicate and results presented as mean ± SE.

Nephrotoxicity was determined by measuring BUN levels. Nephrotoxicity induced by $Cu^{2+}(NTA)_2$ was dose-dependent (Figure 3.13b) and was completely prevented by the administration of ammonium tetrathiomolybdate, desferoxamine and leupeptin. DMSO failed to protect, while excess GSH appeared to enhance the nephrotoxicity of $Cu^{2+}(NTA)_2$ in CD1 mice (Figure 3.15). Experiments were performed in triplicate and results are represented as mean ± SE.

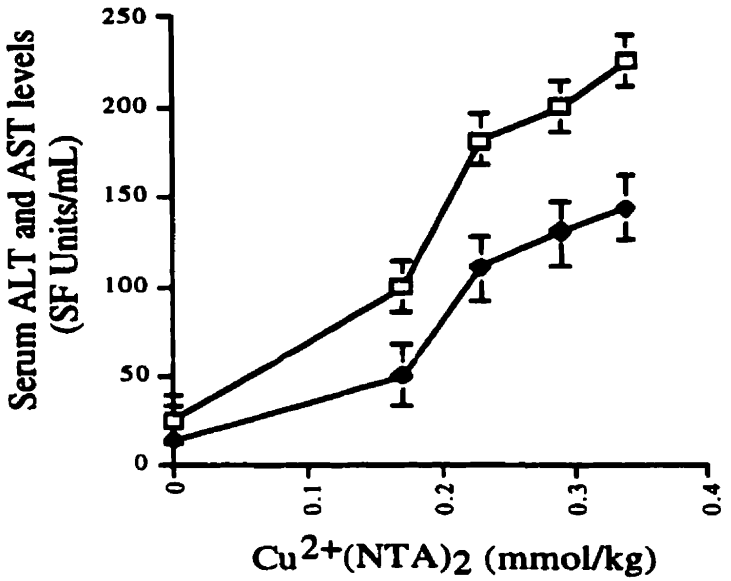


Figure 3.13a : Dose dependent hepatotoxicity at 24 hours after administration of $Cu^{2+}(NTA)_2$ to CD1 mice. Hepatotoxicity was determined by measuring the levels of serum transaminases, ALT (◆) and AST (□) (SF Units/mL). Values represent mean ± SE of three separate experiments. Each group of control/treated animals consisted of 2-3 mice.

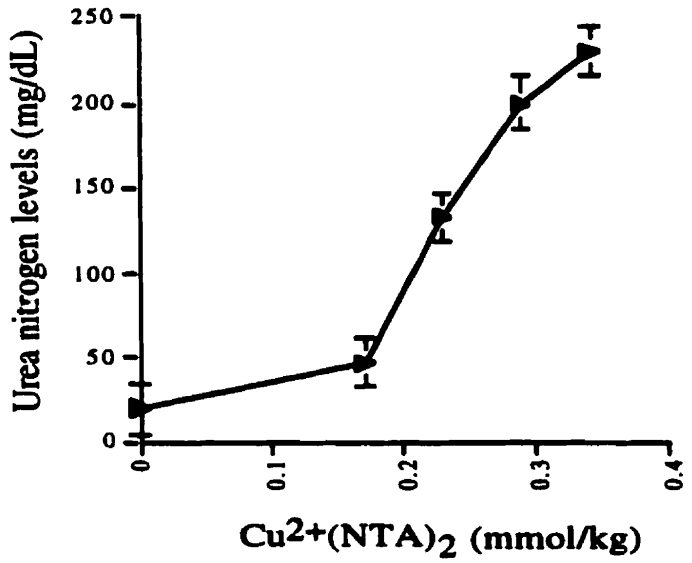


Figure 3.13b: Dose dependent nephrotoxicity after 24 hours administration of $Cu^{2+}(NTA)_2$ in CD1 mice. BUN levels (◗) (mg/dL) was indicator used to determine extent of kidney damage. Values represent mean ± SE of three separate experiments. Each group of control/treated animals consisted of 2-3 mice.

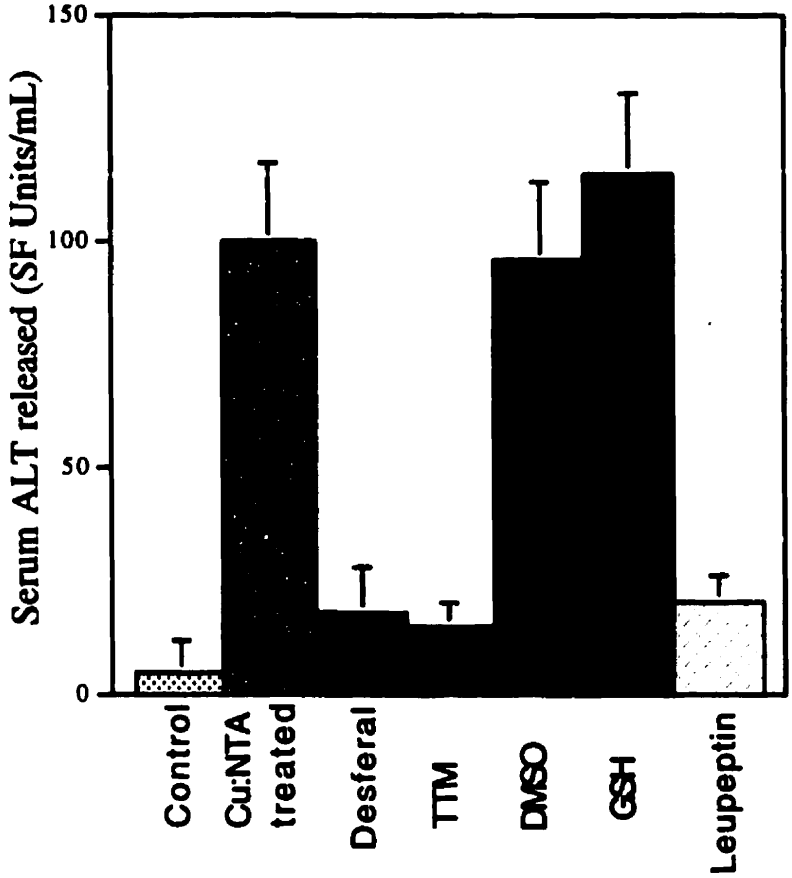


Figure 3.14a: Serum ALT (alanine-αKG aminotransferase) determined from $Cu^{2+}(NTA)_2$ treatment of CD1 mice. Control mice were untreated and injected i.p. with saline instead. The $Cu^{2+}(NTA)_2$ dose used was 0.23 mmol/kg i.p. for all treatments. Doses of modulators administered were as follows:- Desferoxamine (desferal) 35mg/kg , ammonium tetrathiomolybdate (TTM) (10mg/kg), DMSO (4mL/kg), GSH in excess (5mmol/kg) and leupeptin (1.0 mg/kg). Each treatment group consisted of 2-3 CD1 mice and values represent mean ± SE of three separate experiments.

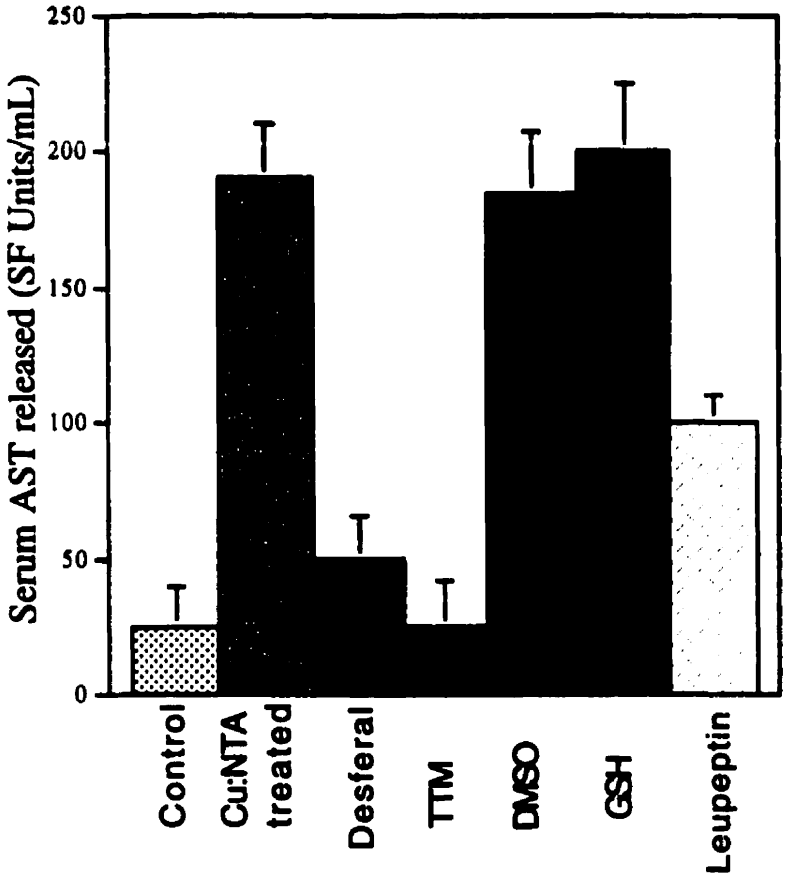


Figure 3.14b: Serum AST (aspartate α-KG aminotransferase) determined from $Cu^{2+}(NTA)_2$ induced treatment of CD1 mice. Control mice were untreated and injected i.p. with saline instead. $Cu^{2+}(NTA)_2$ dose used was 0.23 mmol/kg i.p. for all treatments. Doses of modulators administered i.p. were as follows:- Desferoxamine (desferal) 35mg/kg , ammonium tetrathiomolybdate (TTM) (10mg/kg), DMSO (4mL/kg), GSH in excess (5mmol/kg) and leupeptin (1.0 mg/kg). Each treatment group consisted of 2-3 CD1 mice and values represent mean ± SE of three separate experiments.

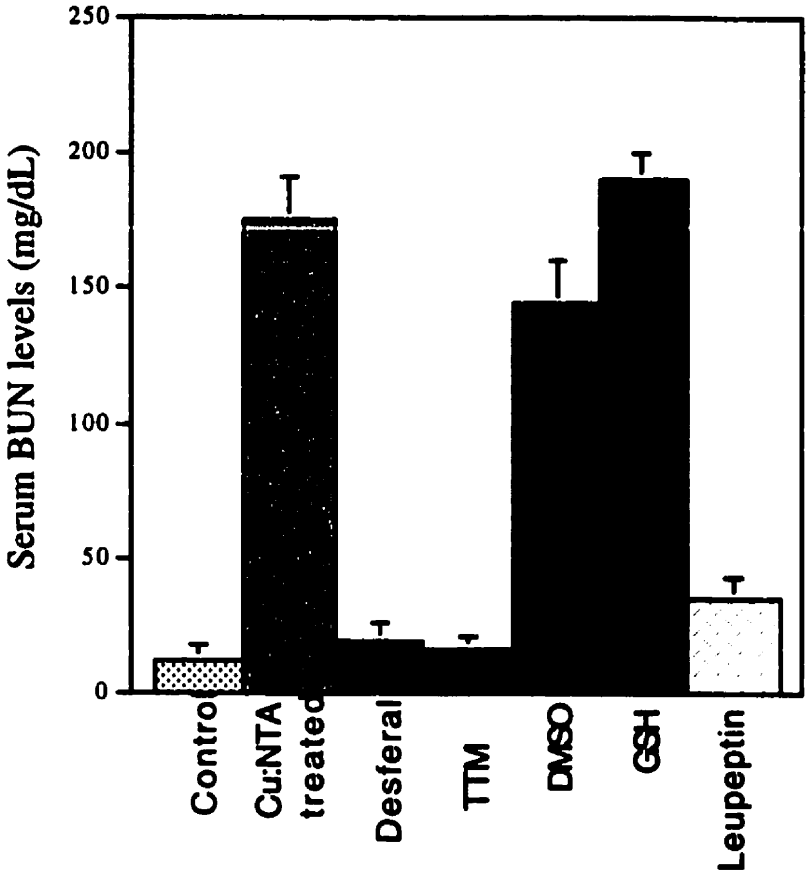


Figure 3.15: Nephrotoxicity induced in $Cu^{2+}(NTA)_2$ treated CD1 mice and modulated with various agents. Control mice were injected with saline. $Cu^{2+}(NTA)_2$ dose used was 0.23 mmol/kg i.p. for all treatments. Doses of modulators administered i.p. were as follows:-Desferoxamine (desferal) 35mg/kg , ammonium tetrathiomolybdate (TTM) (10mg/kg) (Suzuki et al., 1995), DMSO (4mL/kg), GSH in excess (5mmol/kg) and leupeptin (1.0 mg/kg). Each treatment group consisted of 2-3 CD1 mice and values represent mean ± SE of three separate experiments.

3.3. DISCUSSION

In this present study, it was observed that overloading isolated hepatocytes with iron or copper by incubating with $Cu^{2+}(NTA)_2$ or $Fe^{3+}(NTA)_2$ respectively, resulted in a 50-70% loss in mitochondrial function, with loss of free protein sulfhydryl ligands and some depletion / oxidation of GSH. Copper loading with $Cu^{2+}(NTA)_2$ in hepatocytes was 5-6 fold more toxic than iron loading with $Fe^{3+}(NTA)_2$. It was also observed that copper loading toxicity was prevented largely by ROS scavengers whereas that of iron loading was prevented by antioxidants. This suggested that despite the same endpoint in mitochondrial damage, leading to loss in cell viability, both iron and copper have different toxicity initiating mechanisms. Thus iron toxicity was more dependent upon significant lipid peroxidation and copper toxicity was related to the generation of ROS. Nevertheless, the reason(s) for copper loading being more toxic than iron loading are not fully understood at this time.

It is widely known that incubation of unsaturated phospholipids with iron induces lipid peroxidation (Minotti and Aust, 1989). Ferrous iron and oxygen are also able to promote the formation of ROS via the Haber-Weiss and Fenton reactions (Liochev and Fridovich, 1994). Nonetheless, the relationship between the extent of peroxidative damage and cell death has not been clearly established. Morel et al. (1990) demonstrated with cultured hepatocytes that ferric iron complexed with nitrilotriacetate not only stimulated lipid peroxidation but also caused the leakage of intracellular enzymes as a result of irreversible cell injury. Carini et al. (1992) confirmed these findings and showed

that the oxidative damage induced by $Fe^{3+}(NTA)_2$ involved only minor decreases in GSH and little changes in protein thiols even though Fe^{3+} supposedly forms autooxidizable Fe^{2+}-GSH complexes with GSH (Hamed et al., 1983). NTBI iron including $Fe^{3+}(NTA)_2$ are imported into hepatocytes by a low K_M iron transporter, identified by Barisani et al. (1995), having a V_{max} of 241pmol/10^6 cells/min for $Fe^{3+}(NTA)_2$ (0.1-5μM). This finding suggests the role of NTA as an iron loading agent as well as reflect on the toxicology of NTA as a metal-chelating agent due to the uptake by hepatocytes. In another report, the oral LD_{50} of NTA (g/kg) found was 5.34, while chelation to Cu^{2+} and Na^+ greatly reduced the oral LD_{50} to 0.81 g/kg. Nevertheless, chelation to Zn^{2+} or Ca^{2+} or Ni^{2+} increased the oral LD_{50} to 20 g/kg or higher (CRC Crit. Rev. Tox.). Therefore, the toxicology of NTA is very much dependent on the bound metal.

Previous investigators have proposed that cytotoxicity was preceeded by extensive mitochondrial lipid peroxidation, a decrease of mitochondrial transmembrane potential and hepatocyte ATP depletion. However, the reactive species responsible for irreversible cell injury were not identified. Furthermore, GSH-depleted hepatocytes were more susceptible to $Fe^{3+}(NTA)_2$ induced lipid peroxidation and cytotoxicity. Here, we have confirmed that $Fe^{3+}(NTA)_2$ caused rapid lipid peroxidation well before changes in mitochondrial membrane potential and cytotoxicity ensued. The lipid peroxidation contributes to cell death as hepatocytes were completely protected by antioxidants, DPPD and BHA. Surprisingly, ROS scavengers, namely catalase and SOD; the thiol

reductant, DTT; and the •OH scavengers, DMSO and mannitol failed to protect hepatocytes significantly against iron overload cytotoxicity.

Bacon et al. (1993) had earlier identified that chronic iron overload resulted in the partial inactivation of cytochrome c oxidase (Complex IV) in the mitochondrial electron transport chain, resulting in a 48% decrease in respiration with a concomittant 40% decrease in hepatic ATP levels (Bacon et al., 1993). Cytochrome c oxidase activity is dependent upon the existence of intact phospholipids, especially, cardiolipin. Unlike other phospholipids (phosphatidylcholine and phosphatidylethanolamine), cardiolipin contains a higher percentage of polyunsaturated fatty acids (PUFA) and is thus more susceptible to peroxidative injury (Gutteridge et al., 1987). Reactive aldehydic products of lipid peroxidation may also form adducts with lysine residues of cytochrome c oxidase, modify the covalent structure of the enzyme and thus interfere with electron flow (Morel et al., 1990).

However, there was insignificant protection against $Fe^{3+}(NTA)_2$ hepatocyte cytotoxicity from ROS scavengers, namely SOD, catalase, DMSO and mannitol. It was therefore more likely that perpetuation of membrane lipid peroxidative reactions involved the redox cycling of higher oxidation states of "free" iron, rather than •OH radicals. This would explain why desferoxamine, a therapeutic ferric chelator completely prevented $Fe^{3+}(NTA)_2$ toxicity and lipid peroxidation. Subsequent iron-induced peroxidative damage to endosomes and lysosomes may also initiate the release of stored iron from ferritin or hemosiderin, which in turn could generate sufficient ROS to overwhelm the defense

capacity of the hepatocyte. Desferoxamine can be endocytosed by hepatocytes and would therefore be particularly effective against iron released in the lysosomes and endocytotic vacuoles (Ollinger and Brunk, 1995).

Copper overload with Cu^{2+} $(NTA)_2$, however, was more dose-effective than its iron counterpart. Furthermore, protein thiols were depleted and there was also much more GSH depletion as well as GSSG formation. Probably the most important difference between Cu^{2+} and Fe^{3+} overload was that in the case of Cu^{2+} overload, there was less lipid peroxidation. Furthermore antioxidants, such as BHA and DPPD prevented $Fe^{3+}(NTA)_2$ cytotoxicity but failed to protect hepatocytes from Cu^{2+} $(NTA)_2$. However, the •OH radical scavengers, DMSO and mannitol, protected the hepatocytes from Cu^{2+} $(NTA)_2$ but not $Fe^{3+}(NTA)_2$ without preventing lipid peroxidation. Furthermore, catalase and SOD, which scavenge H_2O_2 and $\bullet O_2^-$,respectively, were also effective in preventing Cu^{2+} $(NTA)_2$ but not $Fe^{3+}(NTA)_2$ cytotoxicity.

Another point of difference of Cu^{2+} $(NTA)_2$ from $Fe^{3+}(NTA)_2$ induced cytotoxicity was that GSH depleted hepatocytes were more susceptible to Cu^{2+} $(NTA)_2$. It was recently reported that Cu^{2+} and Cu^{1+} form a strong Cu^{1+}-GSH complex (Goren et al., 1996). The structure of the complex is polymeric with thiolate sulfurs bridging between Cu^{1+} ions. GSH could detoxify Cu^{2+} and prevent oxygen activation by transporting and inserting Cu^{1+} into metallothionein, superoxide dismutase and cytochrome oxidase (Vulpe and Packman, 1995). This could explain the increase in the susceptibility of hepatocytes to copper if GSH was depleted beforehand. DTT, the thiol

reductant, also prevented cytotoxicity even if added sometime after $Cu^{2+}(NTA)_2$ probably as a result of reducing oxidized protein sulfhydryls and/or chelating Cu^{2+}. A common belief is that the Cu^{1+}-GSH complex is the source of cytotoxic ROS and free Cu^{2+} (Goren et al., 1996; Morel et al., 1983) as shown in the following equation:

$$Cu^{1+}\text{-}GS^- + O_2 \Longrightarrow GS^\bullet + Cu^+ + \bullet O_2^-$$

However, Cu^{1+}-GSH in contrast to the Cu^{1+}.cysteine complex is relatively stable under aerobic conditions and oxygen activation is markedly increased 6-10 fold in GSH depleted hepatocytes treated with Cu^{2+}. It is more likely that GSH detoxifies Cu^{2+} but can release its Cu^{1+} at higher Cu^{2+} concentrations. The release of Cu^{1+} is predicted in the following equation: $Cu^{1+}\text{-}GS^- + Cu^{2+} \Longrightarrow GS^\bullet + 2Cu^+$

The increased susceptibility of hepatocytes at low oxygen concentrations (1% vs. 95% O_2) to both $Cu^{2+}(NTA)_2$ and $Fe^{3+}(NTA)_2$ can be explained if Cu^{2+} /Fe^{3+} overloading caused mitochondrial damage, which resulted in impaired respiration and subsequent loss in ATP generation and membrane potential. Previously our research group demonstrated that hepatocytes are more susceptible to mitochondrial respiratory inhibitors at low oxygen concentrations (Niknahad and O' Brien, 1995). It should be noted with caution that the cytoprotective effect of ruthenium red may not indicate that cytotoxicity involves futile Ca^{2+} cycling in mitochondria. In our experiments, ruthenium red also completely prevented lipid peroxidation from $Cu^{2+}(NTA)_2$. Other investigators have

suggested ruthenium red can act as an antioxidant (Bacon et al., 1993). Consequently, its protective effect may not reflect its effect on Ca^{2+} cycling.

To find a chelation therapy which would prevent hepatocyte cytotoxicity resulting from copper overload, the effects of adding D-penicillamine, ammonium tetrathiomolybdate or desferoxamine after $Cu^{2+}(NTA)_2$ were investigated. A marked protection against $Cu^{2+}(NTA)_2$ cytotoxicity was observed with TTM and D-penicillamine. D-penicillamine has been a therapy for copper overloading since the 1970's until recently, when studies revealed the potential risk of penicillamine therapy in developing neurological and autoimmune disorders after prolonged drug intake (Brewer et al., 1987). TTM is currently used to treat Cu^{2+} -poisoned farm animals (Gooneratne et al., 1981) and has recently been used in clinical trials to treat the genetic disorder Wilson's disease. It should prove useful for 20-30% of patients who develop hypersensitivity reactions or neurological disorders following penicillamine treatment. TTM blocks the absorption of Cu^{2+} and reacts selectively and directly with copper, either bound to MT or "free" to form the nontoxic Cu/TTM complex as identified by Ogra et al. (1996). D-penicillamine acts by reductive chelation of copper from the cellular pool and the Cu^{1+} chelate effluxes the cell. It commonly causes a large initial negative copper balance (Walshe, 1956). It was most interesting that desferoxamine, a ferric chelator (log K = 30.6), markedly prevented $Cu^{2+}(NTA)_2$ toxicity and lipid peroxidation. This suggests that endogenous iron plays a role in Cu^{2+} catalyzed lipid peroxidation and cytotoxicity or that desferoxamine chelates Cu^{2+} (log K = 14.0).

Protease inhibitors have recently been introduced as a cotherapy for AIDS. E64 is a lysosomal cysteine proteinase inhibitor, that prevents the degradative action of lysosomal enzymes released upon membrane destabilization (Montenez et al., 1994; Mego, 1983). It was found that $Cu^{2+}(NTA)_2$ cytotoxicity was markedly prevented by E64. The protection by E64 suggests that copper overload toxicity involves an accumulation of copper in the lysosomes which reacts with intracellular H_2O_2 to form reactive oxygen species. The latter may cause lysosomal membrane damage which subsequently leads to the leakage of cytotoxic proteolytic enzymes. Myers et al. (1993) confirmed that copper overload resulted in copper accumulation in hepatocyte lysosomes which initiated the lipid peroxidation of lysosomal membranes. This led to physicochemical alterations in the lysosomal membrane as observed from the loss in membrane fluidity, increased membrane fragility and altered membrane composition. They suggested that alterations to membrane integrity may contribute to lysosomal protease release or other degradative enzymes.

Another interesting phenomenon from our results was the enhancement of $Cu^{2+}(NTA)_2$ cytotoxicity by cysteine which was more than that seen with $Fe^{3+}(NTA)_2$ cytotoxicity. This probably suggested that cysteine, as a thiol ligand, either I) assisted in the uptake of copper into hepatocyte or II) reduced the copper which caused the generation of substantial amounts of ROS, namely superoxide, hydrogen peroxide, and •OH production that oxidized neighbouring lipid membranes. The role of cysteine as a physiological reductant or transporter has been investigated with $Fe^{3+}(NTA)_2$ on kidney

tissue culture. It was found that nephrotoxicity was prevented by acivicin treatment of cells (Okada et al., 1993). Acivicin (AT-125) is an irreversible inhibitor of γ-GTP (Gardell and Tate, 1980). γ-GTP is a cell surface enzyme that cleaves the γ-glutamyl bond of GSH, which degrades the tripeptide to yield cysteinyl-glycine. This dipeptide is then cleaved by the dipeptidase to produce cysteine (Hanigan and Pitot, 1985). The prevention of $Fe^{3+}(NTA)_2$ induced nephrotoxicity by acivicin thus suggested that cysteine, being a product of the degradation of GSH by the kidney may activate Cu^{2+} $(NTA)_2$ *in vivo* (Hamazaki et al, 1989). However, the administration of cysteine or GSH *in vivo* inhibited $Fe^{3+}(NTA)_2$ induced 8-OH deoxyguanosine formation in rats (Umemura et al., 1991).

Although various investigators have demonstrated *in vivo* hepato and nephrotoxicity following the i.p. administration of an acute dose of $Fe^{3+}(NTA)_2$, such studies have not been reported for Cu^{2+} $(NTA)_2$. Therefore, we proceeded to an *in vivo* study of Cu^{2+} $(NTA)_2$ toxicity in CD1 mice. It was found that 24 hours after an acute dose of Cu^{2+} $(NTA)_2$ there was an 8-fold increase in serum levels of ALT, AST and BUN relative to control mice was formed. These parameters are indicative of liver and renal damage. This damage, however, did not occur if the mice were also treated with the ferric chelator, desferoxamine and the copper chelator, TTM. However, the •OH radical scavenger, DMSO injected intraperitoneally was ineffective against Cu^{2+} $(NTA)_2$ induced hepato and nephrotoxicity.

The administration of reduced GSH 30 minutes prior to Cu^{2+} $(NTA)_2$ injection slightly increased hepato and nephrotoxicity possibly as a result of increasing plasma cysteine levels. GSH is rapidly metabolised to cysteine by γ-GTP and dipeptidase localized on the luminal surface of the proximal tubule cells where it has access to all components of the glomerular filtrate (Glenner et al., 1962). The mice either suffered from severe renal and liver damage or failed to survive the 24 hours following the Cu^{2+} $(NTA)_2$ injection. Plasma cysteine could also contribute to the uptake of copper by liver and kidney besides causing redox cycling and oxygen activation by copper.

In short, both copper and iron overload of hepatocytes with Cu^{2+} $(NTA)_2$ and $Fe^{3+}(NTA)_2$ complexes appear to lead to similar endpoints of toxicity, yet the toxicity initiation mechanisms differ considerably. Copper overload toxicity at least in isolated hepatocytes is mediated by reactive oxygen species and is prevented by ROS scavengers, whereas iron overload toxicity in isolated hepatocytes is mediated by lipid peroxidation and is prevented by antioxidants.

Chapter 4

THE CYTOTOXIC MECHANISMS OF COPPER COMPLEXES OF SELECTED PHENANTHROLINE DERIVATIVES

4.1. INTRODUCTION

Copper chelates with phenanthroline ligands, namely 1,10-phenanthroline (OP) and 2,9-dimethyl-1,10-phenanthroline (neocuproine, NC) have been investigated as possible anticancer agents. Treatment of Ehrlich ascites tumor cells with $Cu^{2+}(OP)_2$ (2nmol/10^5 cells) or $Cu^{2+}(NC)_2$ (0.05nmol/10^5 cells) for 48 hours inhibited growth by 50% (Brynes et al., 1992). Furthermore, OP and NC have also been used to prevent oxidative stress cytotoxicity to indicate whether intracellular iron or copper respectively catalysed the oxidative stress (Filho and Meneghini, 1985). OP prevented the inhibition of DNA synthesis by the antitumor drug, bleomycin, thereby implicating the role of iron in the bleomycin toxicity mechanism (Gutteridge and Halliwell, 1989); NC protected isolated rat hearts against H_2O_2 and ischemia/reperfusion induced injury (Appelbaum et al., 1989).

The $Cu^{2+}(OP)_2$ complex is reduced by cysteine or ascorbic acid to form $Cu^{1+}(OP)_2$ which is highly autoxidisable. The $Cu^{2+}(OP)_2$ complex acts as a "chemical nuclease" towards DNA *in vitro* as a result of the formation of active oxygen species (Brynes et al., 1992, Burkitt et al., 1996) by the following reactions (Burkitt et al., 1996):

$$Cu^{1+}(OP)_2 + O_2 <=> \bullet O_2^- + Cu^{2+}(OP)_2$$

$$Cu^{1+}(OP)_2 + \bullet O_2^- <=> H_2O_2 + Cu^{2+}(OP)_2$$

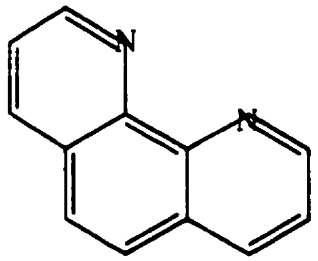
$$Cu^{1+}(OP)_2 + H_2O_2 <=> \bullet OH + OH^- + Cu^{2+}(OP)_2$$

NC and BC, form much more stable coordination complexes with Cu(I) than Cu(II). The structure and partition coefficients of the OP analogues are shown in Figure 4.1. The stability constants and redox potentials of the copper complexes are also included from reference tables (James and Williams, 1961). The $Cu^{1+}(NC)_2$ complex has a high reduction potential, is highly stable under aerobic conditions and would not be expected to activate oxygen by redox cycling between the Cu(I) and Cu(II) state. Recent experiments, have proven that $Cu^{2+}(OP)_2$ is much more potent than $Cu^{2+}(NC)_2$ in acting as a chemical nuclease (Sigman et al., 1993) or chemical protease (Kito et al., 1995) thereby causing nucleic acid or protein cleavage. It is therefore surprising that $Cu^{2+}(NC)_2$ was more effective than $Cu^{2+}(OP)_2$ in preventing the growth of Ehrlich ascites tumor cells (Brynes et al., 1992).

This raised the question of whether redox cycling was essential for the cytotoxic mechanism of copper complexes and how the nature of the metal chelator can affect the cytotoxic nature of copper. Each chelator varies the redox-potential of the metal. Steric effects may also determine which intracellular target molecules or reductive enzymes interact with the metal complex (Chevion, 1988). Moreover, the nature of the chelator would also markedly determine the partition effect of the metal complex in lipid and

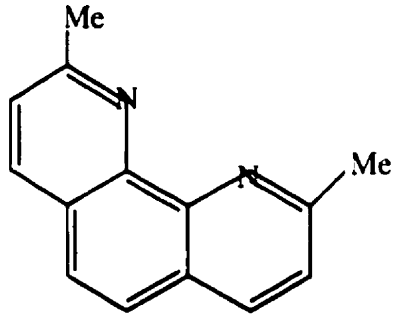
aqueous phases, and thereby determine the rate of copper uptake and the specificity for reductive enzymes or target molecules. The partition of Cu^{2+} into *n*-octanol from an aqueous solution is increased 6-fold if chelated with OP (Ou et al., 1995). Chelation with NC was even more effective than OP at increasing the uptake of Cu into cells (Brynes et al., 1992).

The objective of this present study was to compare and contrast the differences in cytotoxic mechanisms of copper [Cu^{2+}] with the complexes of two selected phenanthroline derivatives, namely $Cu^{2+}(OP)_2$ and $Cu^{2+}(NC)_2$, by comparing the changes in cellular GSH and GSSG levels with their effectiveness at activating oxygen or causing lipid peroxidation. The involvement of these changes in the cytotoxic pathways were investigated with radical scavengers, antioxidants and ROS scavenger enzymes. These results would then reflect on the significance and role of chelating agents in copper toxicity. In this study, we present evidence of contrasting effects between $Cu^{2+}(OP)_2$ and $Cu^{2+}(NC)_2$ in their effects on hepatocytes but which ultimately leads to a common pathway resulting in cell death.



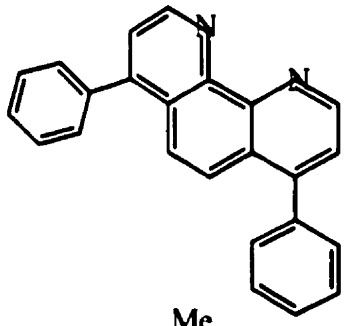
1,10-phenanthroline $P_{oct/buffer} = 2.8$

$Cu^{2+}(OP)_2$ stability constant = 9.25
E_o=110mV; $P_{oct/buffer} = 0.024$



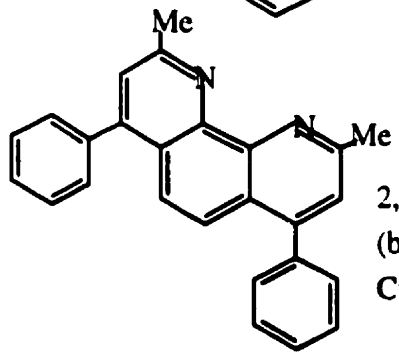
2,9-dimethyl-1,10-phenanthroline (neocuproine; NC)

$Cu^{2+}(NC)_2$ stability constant = 6.1 ; E_o= 594mV
$Cu^{1+}(NC)_2$ stability constant = 19.1



4,7-diphenyl-1,10-phenanthroline
(bathophenanthroline, BP); $P_{oct/buffer}$= 26

$Cu^{2+}(BP)_2$ stability constant = 5.7;
E_o = 230mV ; $P_{oct/buffer} = 0.78$



2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline
(bathocuproine, BC)
$Cu^{2+}(BC)_2$ E_o = 400mV

Figure 4.1: Properties of 1,10-phenanthroline analogues and their copper complexes

4.2. RESULTS

4.2.1. Enhanced Cytotoxicity in Hepatocytes by copper complexes

As shown in Figure 4.2, the cytotoxicity of copper was increased upon complexation with the chelators, NC and OP respectively. Unchelated copper ($30\mu M/10^6$ cells) was thus less cytotoxic than the copper complexes, $Cu^{2+}(NC)_2$ ($20\mu M/10^6$ cells) and $Cu^{2+}(OP)_2$ ($8\mu M/10^6$ cells) in isolated rat hepatocytes. Cell lysis with $Cu^{2+}(NC)_2$ ($20\mu M/10^6$ cells) steadily increased over time whereas cell lysis with $Cu^{2+}(OP)_2$ ($8\mu M/10^6$ cells) may occur after a slight lag. The cytotoxicity of the chelators in the absence of Cu^{2+} was also determined. The concentration of OP required to cause 50% cytotoxicity in 2 hrs was approximately $300\mu M/10^6$ cells whereas the concentration of NC required to cause 50% cytotoxicity was $500\mu M/10^6$ cells.

Lipid peroxidation was increased 3-fold by $Cu^{2+}(OP)_2$ ($8\mu M/10^6$ cells) whereas $Cu^{2+}(NC)_2$ ($20\mu M/10^6$ cells) did not cause significant lipid peroxidation (Figure 4.3). Unchelated copper ($30\mu M/10^6$ cells) caused relatively less lipid peroxidation than $Cu^{2+}(OP)_2$. It was interesting that $Cu^{2+}(NC)_2$ may act as an antioxidant as lipid peroxidation was not induced.

In Figure 4.4a, $Cu^{2+}(NC)_2$ was more effective than $Cu^{2+}(OP)_2$ at depleting GSH. Unchelated copper ($30\mu M/10^6$ cells) merely depleted 10-20% of control GSH levels. GSH depletion by $Cu^{2+}(NC)_2$ was accompanied by GSSG formation. However, this was

prevented if 150μM cystine was present (results not shown). Hepatocytes are impermeable to cystine and thus it is more likely that $Cu^{2+}(NC)_2$ caused GSH efflux from the hepatocytes which subsequently autooxidized in the medium. On the other hand, the smaller amount of GSSG formation caused by $Cu^{2+}(OP)_2$ reached a plateau after the first 0.5 hours but was not affected by cystine (results not shown). Unchelated copper did not significantly increase GSSG levels (Figure 4.4b).

4.2.2. Modulation of cytotoxicity by copper complexes

As shown in Figure 4.5, $Cu^{2+}(NC)_2$ (20μM/10^6 cells) cytotoxicity was prevented with SOD (20μg/10^6 cells) and the cell permeable dithiol, dithiothreitol (DTT) despite blebbing of the plasma membrane. Neither the extracellular cuprous chelator, BCS (50μM/10^6 cells), a derivative of BC with two negatively charged sulfonate ligands nor added catalase (20U/10^6 cells) prevented $Cu^{2+}(NC)_2$ cytotoxicity, but did prevent Cu^{2+}NTA cytotoxicity. The •OH radical scavenger, DMSO (100μL/10^6 cells) also failed to prevent toxicity after 1.5 hours of incubation. Lipid peroxidation by $Cu^{2+}(NC)_2$ was insignificant and antioxidants also did not affect cytotoxicity.

In Figure 4.7, $Cu^{2+}(OP)_2$-induced cytotoxicity was also prevented by SOD (20μg/10^6 cells) and DTT (3mM/10^6 cells). However, the extracellular cuprous chelator, BCS (50μM/10^6 cells) or catalase (20U/10^6 cells) prevented $Cu^{2+}(OP)_2$ cytotoxicity as did cyanide (20μM/10^6 cells). Furthermore, DMSO (100μL/10^6 cells) afforded initial protection for 1.5 hours. Marked lipid peroxidation occurred but as shown in Figure 4.8,

DPPD (1μM/10^6 cells), a potent antioxidant, failed to prevent cytotoxicity. This suggests that lipid peroxidation may not contribute to irreversible cell death. DTT, BCS and cyanide prevented lipid peroxidation in treated rat hepatocytes whereas catalase and DMSO failed to prevent lipid peroxidation. GSH depletion by $Cu^{2+}(NC)_2$ was greatly prevented with DTT, but not with catalase or BCS but slightly with SOD (Figure 4.6). In Figure 4.9, GSH depletion by $Cu^{2+}(OP)_2$ was prevented with catalase, BCS and DTT. Furthermore, GSH-depleted hepatocytes (with 1-bromoheptane) were more susceptible to $Cu^{2+}(NC)_2$ or $Cu^{2+}(OP)_2$ as shown in Figure 4.10.

In Figure 4.11a, it was observed that $Cu^{2+}(OP)_2$ (8μM/10^6 cells) toxicity was decreased 2-fold by reducing oxygen levels from 95% to 1% O_2 whereas $Cu^{2+}(NC)_2$ (20μM/10^6 cells) toxicity was unaffected. Lipid peroxidation induced by $Cu^{2+}(OP)_2$ was significantly decreased under 1% O_2/ 5% CO_2/ 94% N_2 incubation as shown in Figure 4.11b. By contrast, the cytotoxicity of unchelated copper (30μM/10^6 cells) was increased when oxygen levels were decreased from 95% to 1% O_2. In Figure 4.12, a Clarke oxygen electrode was used to show that $Cu^{2+}(OP)_2$ (8μM/10^6 cells) but not $Cu^{2+}(NC)_2$ (20μM/10^6 cells) markedly induced antimycin-resistant respiration when added to hepatocytes.

Spectroscopic time-studies were performed on the ability of intact hepatocytes to reduce $Cu^{2+}(OP)_2$ and $Cu^{2+}(NC)_2$. Figure 4.13 depicts the reduction of $Cu^{2+}(NC)_2$ (20μM/10^6 cells) and $Cu^{2+}(OP)_2$ (8μM/10^6 cells) when added to isolated hepatocytes.

The rate of $Cu^{2+}(NC)_2$ reduction was relatively faster than that of $Cu^{2+}(OP)_2$. However, the rate of $Cu^{2+}(NC)_2$ reduction was increased 2-3 fold in GSH depleted hepatocytes (Figure 4.13) compared to in normal hepatocytes. This suggests that $Cu^{1+}(NC)_2$ reacts with intracellular GSH to form a $GS.Cu^{1+}$ complex. In the absence of GSH, reduction occurs via various intracellular NAD(P)H reductases or possibly via protein thiols. By contrast, the $Cu^{1+}(OP)_2$ complex rapidly autoxidises and/or is less reactive with GSH. Figure 4.15 compares the liganding capacities of DTT (50μM), L-cysteine (100μM) and GSH (100μM) towards $Cu^{1+}(NC)_2$ in the absence of hepatocytes. $Cu^{2+}(NC)_2$ was reduced immediately by an equimolar concentration of ascorbate prior to the addition of thiol ligands. The order of thiol chelating effectiveness for removing Cu^{1+} from $Cu^{1+}(NC)_2$ was DTT >> L-cysteine > GSH (results not shown). However, the order of thiol reductive effectiveness for reducing $Cu^{2+}(NC)_2$ was Cys > GSH > DTT (Figure 4.14). Results in Figure 4.16a indicate that GSH partly protected the $Cu^{1+}(NC)_2$ complex from DTT. This suggests the possibility of an intermediate $GS.Cu^{1+}.NC$ complex. Furthermore, GSH partly restored the 454 nm absorbance lost upon adding DTT to $Cu^{1+}(NC)_2$ (Figure 4.16b).

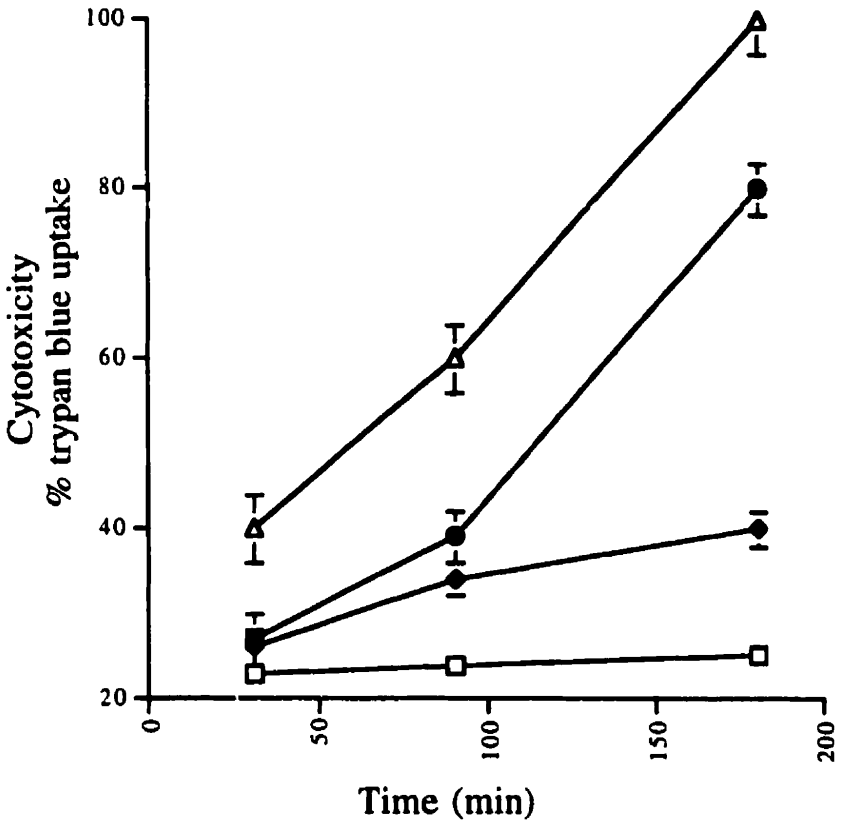


Figure 4.2: Copper chelation increases the toxicity of Cu^{2+} towards isolated hepatocytes. Hepatocytes (1 x 10^6 cells/mL) were incubated with Cu^{2+} (30μM) (◆), $Cu^{2+}(NC)_2$ (20μM) (Δ) and $Cu^{2+}(OP)_2$ (8μM) (●). Control hepatocytes (❑) with no additions.

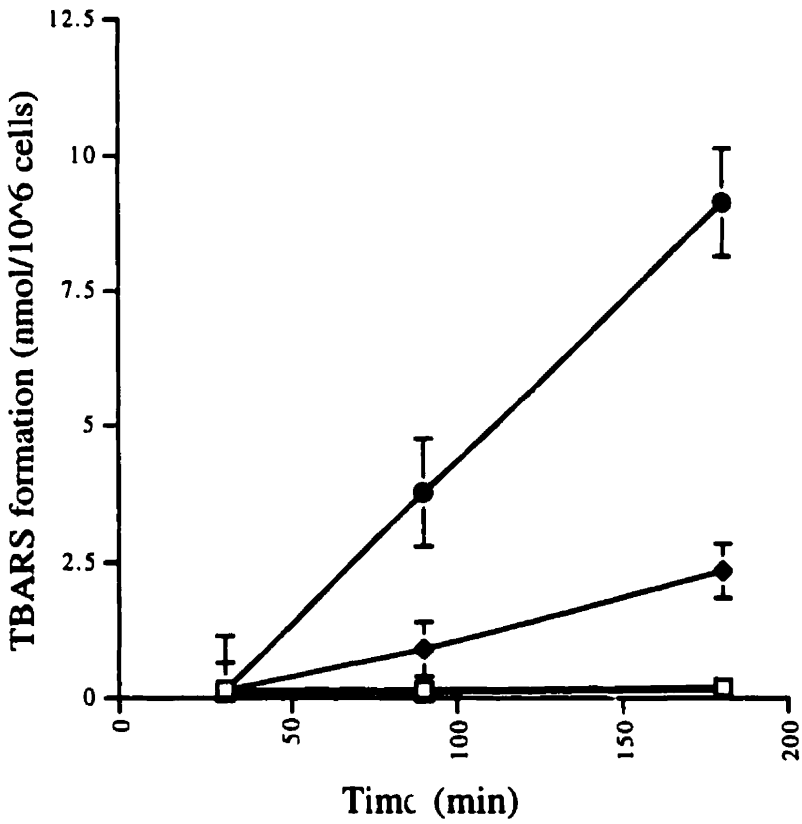


Figure 4.3: Lipid peroxidation induced by copper and copper complexes in isolated hepatocytes. Hepatocytes (1 x 10^6 cells/mL) were incubated with Cu^{2+} (30μM) (◆), $Cu^{2+}(NC)_2$ (20μM) (Δ) and $Cu^{2+}(OP)_2$ (8μM) (●). $Cu^{2+}(OP)_2$ caused significantly more lipid peroxidation than free Cu^{2+} . $Cu^{2+}(NC)_2$ did not cause any detectable lipid peroxidation products. Control hepatocytes (❑) with no additions. Results represent mean ± SE of three separate experiments.

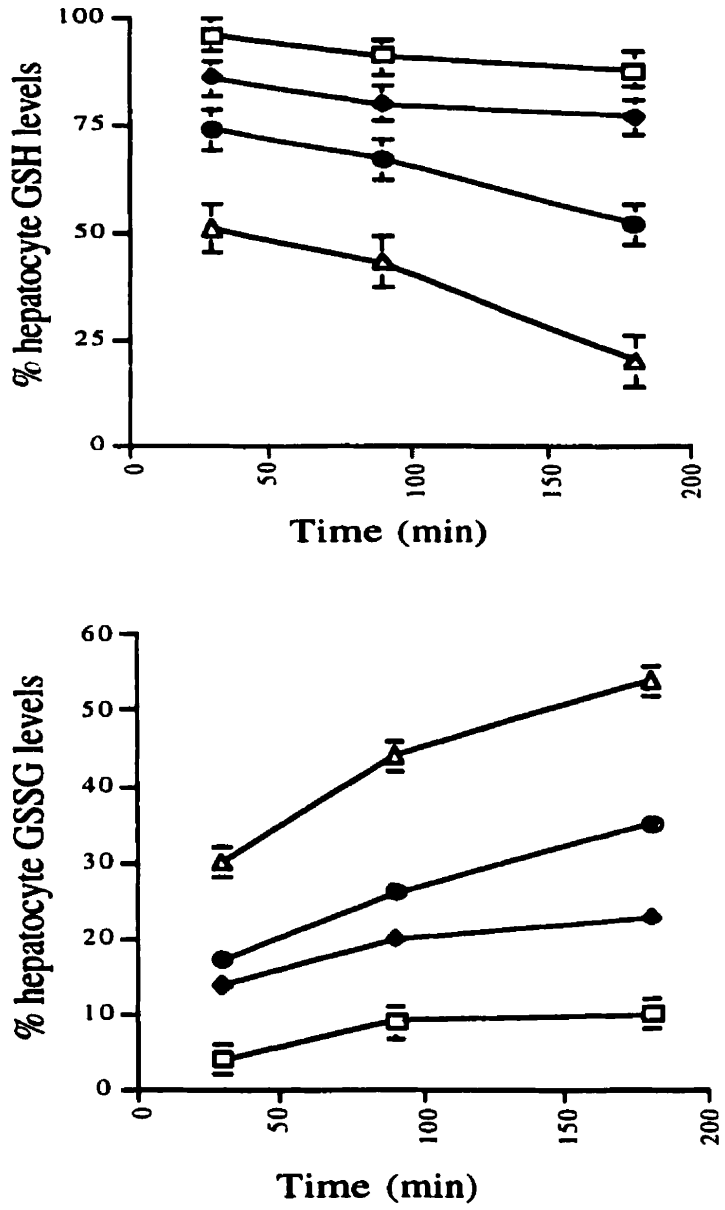


Figure 4.4a and b: Determination of GSH and GSSG in isolated hepatocytes treated with Cu^{2+} , $Cu^{2+}(OP)_2$ and $Cu^{2+}(NC)_2$ .Hepatocytes (1 x 10^6 cells/mL) were incubated with Cu^{2+} (30μM) (◆), $Cu^{2+}(NC)_2$ (20μM) (Δ) and $Cu^{2+}(OP)_2$ (8μM) (●). $Cu^{2+}(NC)_2$ caused significant GSH depletion and formation of GSSG. Control hepatocytes (❑) with no additions. Results represent mean ± SE of three separate experiments.

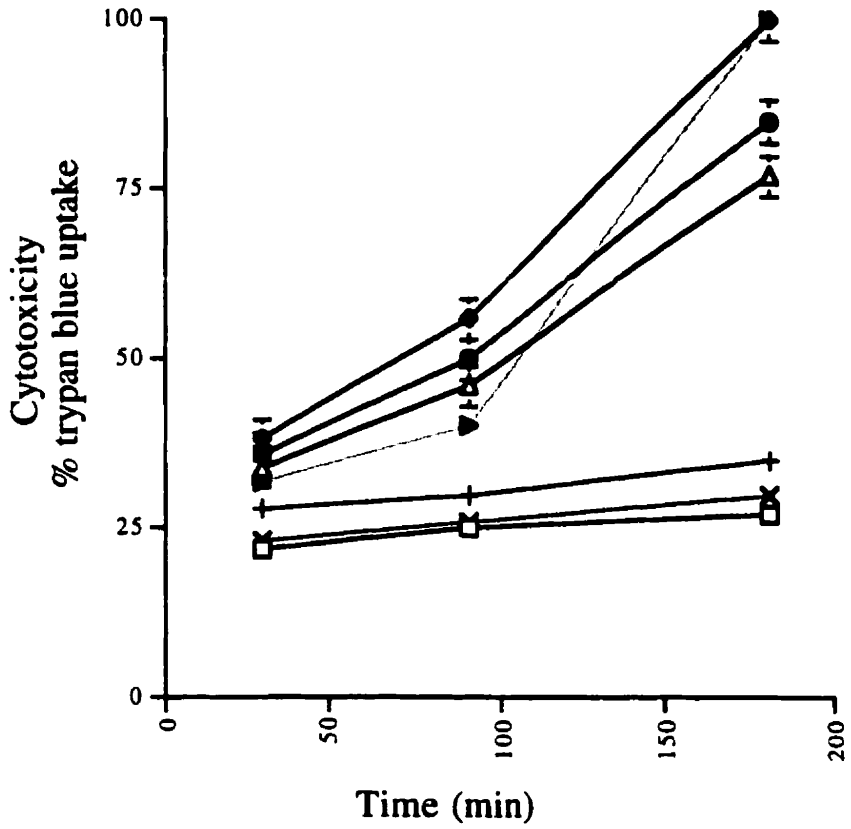


Figure 4.5: Hepatocyte cytotoxicity of $Cu^{2+}(NC)_2$ modulated by different agents in isolated hepatocytes. Hepatocytes (1 x 10^6 cells/mL) were incubated with $Cu^{2+}(NC)_2$ (20μM) (◆), $Cu^{2+}(NC)_2$ + BCS (100μM) (Δ), $Cu^{2+}(NC)_2$ + catalase (20U) (●), $Cu^{2+}(NC)_2$ + DMSO (100μL) (◗), $Cu^{2+}(NC)_2$ + SOD (0.5mg) (+) and $Cu^{2+}(NC)_2$ + DTT (3mM) (X). $Cu^{2+}(NC)_2$ cytotoxicity was prevented with DTT and SOD and delayed initially with DMSO. Catalase and BCS failed to prevent toxicity. Control hepatocytes (❑) with no additions. Results represent mean ± SE of three separate experiments.

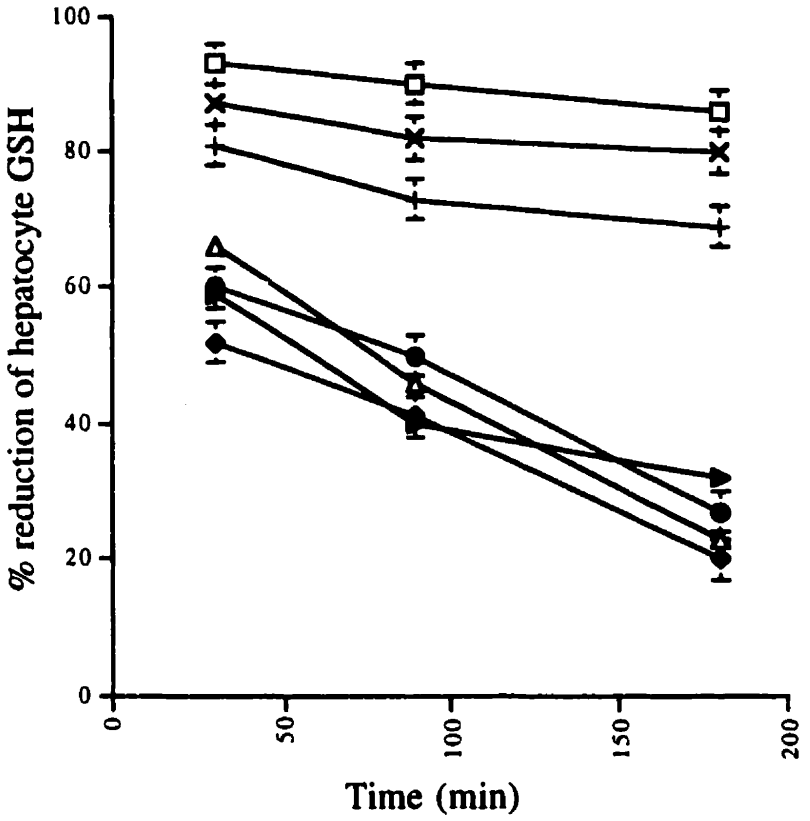


Figure 4.6: Hepatocyte GSH depletion by $Cu^{2+}(NC)_2$ modulated by different agents in isolated hepatocytes. Hepatocytes (1 x 10^6 cells/mL) were incubated with $Cu^{2+}(NC)_2$ (20μM) (◆), $Cu^{2+}(NC)_2$ + BCS (100μM) (Δ), $Cu^{2+}(NC)_2$ + catalase (20U) (●), $Cu^{2+}(NC)_2$ + DMSO (100μL) (◗), $Cu^{2+}(NC)_2$ + SOD (0.5mg) (+) and $Cu^{2+}(NC)_2$ + DTT (3mM) (X). GSH depletion by $Cu^{2+}(NC)_2$ was prevented with DTT and SOD. Catalase, DMSO and BCS failed to prevent GSH depletion. Control hepatocytes (❑) with no additions. Results represent mean ± SE of three separate experiments.

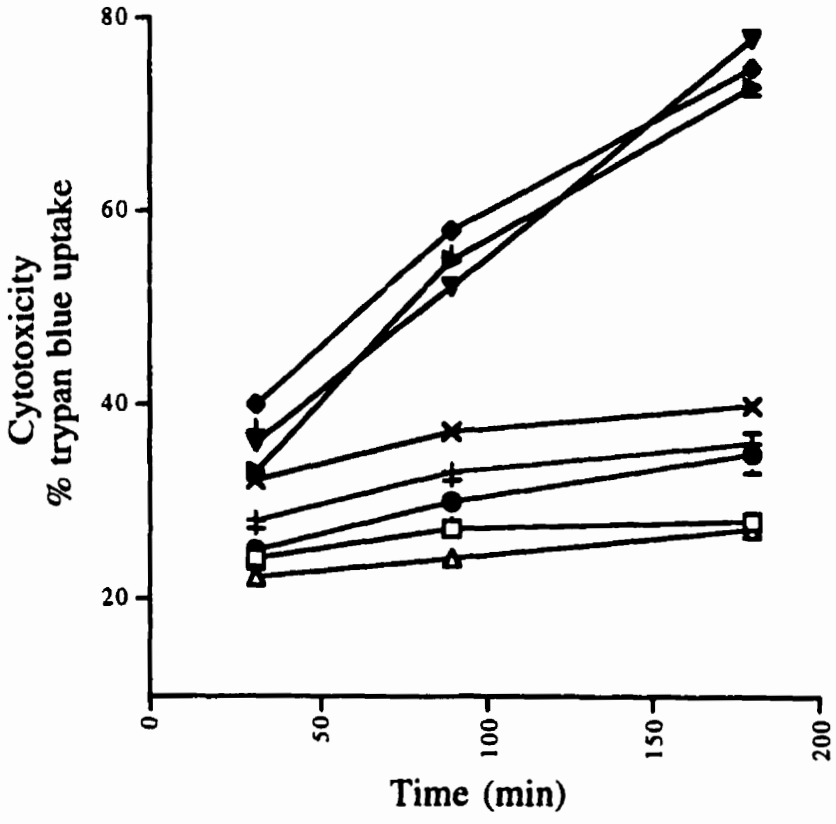


Figure 4.7: Hepatocyte cytotoxicity of $Cu^{2+}(OP)_2$ modulated by different agents in isolated hepatocytes. Hepatocytes (1 x 10^6 cells/mL) were incubated with $Cu^{2+}(OP)_2$ (20μM) (◆), $Cu^{2+}(OP)_2$ + BCS (100μM) (Δ), $Cu^{2+}(OP)_2$ + catalase (20U) (●), $Cu^{2+}(OP)_2$ + DMSO (100μL) (◗), $Cu^{2+}(OP)_2$ + SOD (0.5mg) (+), $Cu^{2+}(OP)_2$ + CN^- (20μM) (○), $Cu^{2+}(OP)_2$ + DPPD (1μM) (▼) and $Cu^{2+}(OP)_2$ + DTT (3mM) (X). $Cu^{2+}(OP)_2$ cytotoxicity was prevented with catalase, SOD, BCS, CN^- and DTT and delayed initially with DMSO. The antioxidant, DPPD failed to prevent toxicity. Control hepatocytes (❑) with no additions. Results represent mean ± SE of three separate experiments.

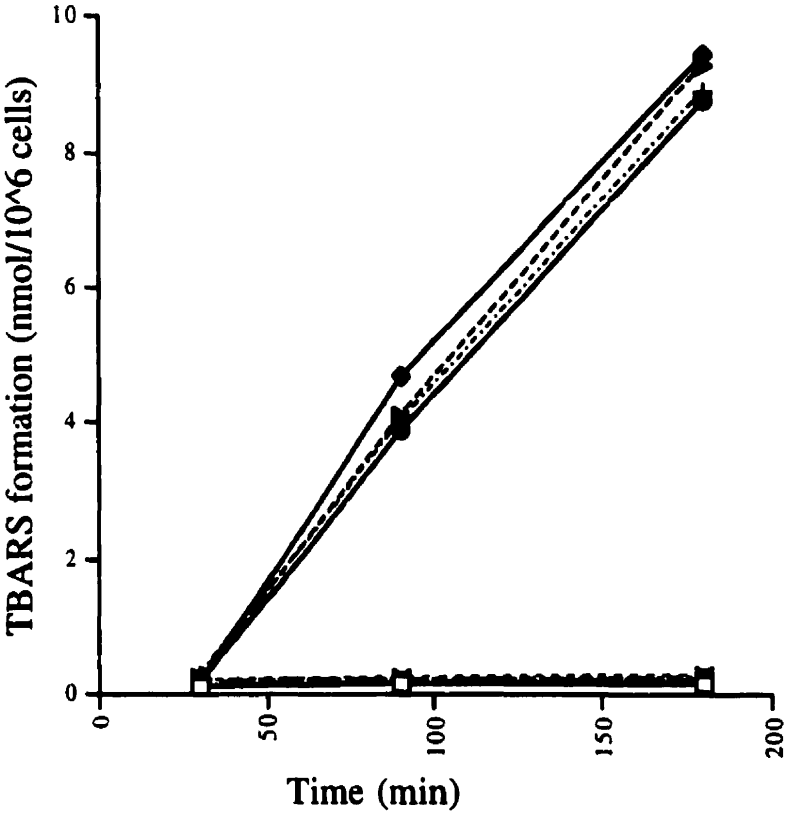


Figure 4.8: Hepatocyte lipid peroxidation induced by $Cu^{2+}(OP)_2$ modulated by different agents in isolated hepatocytes. Hepatocytes (1 x 10^6 cells/mL) were incubated with $Cu^{2+}(OP)_2$ (20μM) (◆), $Cu^{2+}(OP)_2$ + BCS (100μM) (Δ), $Cu^{2+}(OP)_2$ + catalase (20U) (●), $Cu^{2+}(OP)_2$ + DMSO (100μL) (◗), $Cu^{2+}(OP)_2$ + SOD (0.5mg) (+), $Cu^{2+}(OP)_2$ + CN^- (20μM) (○), $Cu^{2+}(OP)_2$ + DPPD (1μM) (▼) and $Cu^{2+}(OP)_2$ + DTT (3mM) (X). Control hepatocytes (❑) with no additions.

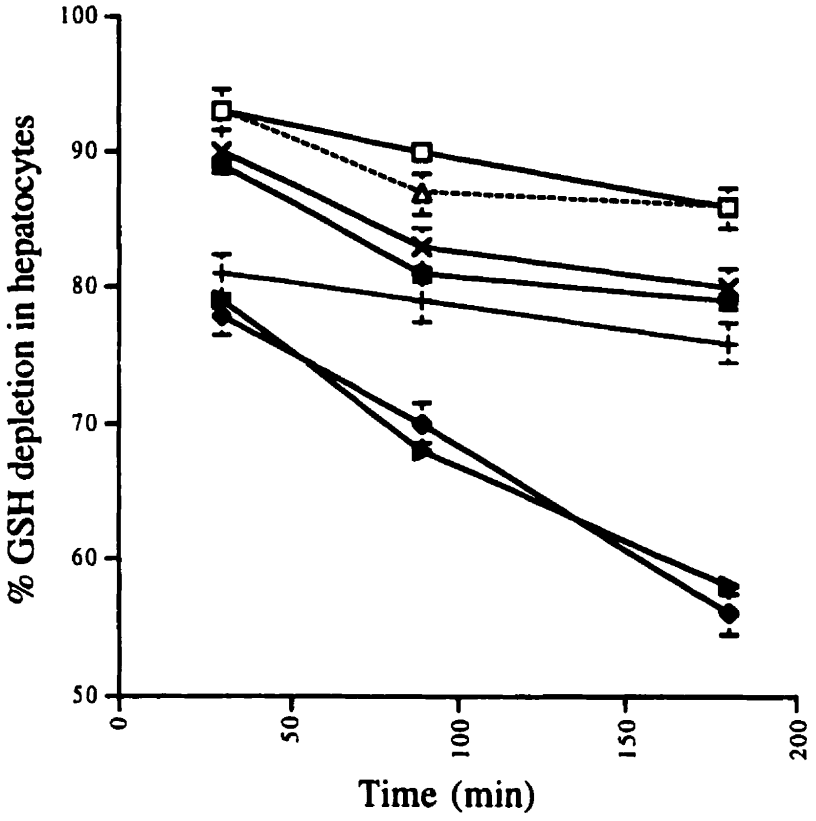


Figure 4.9: Modulation of $Cu^{2+}(OP)_2$ induced GSH depletion by different agents in isolated hepatocytes. Hepatocytes (1 x 10^6 cells/mL) were incubated with $Cu^{2+}(OP)_2$ (20μM) (◆), $Cu^{2+}(OP)_2$ + BCS (100μM) (Δ), $Cu^{2+}(OP)_2$ + catalase (20U) (●), $Cu^{2+}(OP)_2$ + DMSO (100μL) (◗), $Cu^{2+}(OP)_2$ + SOD (0.5mg) (+) and $Cu^{2+}(OP)_2$ + DTT (3mM) (X). Control hepatocytes (❑) with no additions. Results represent mean ± SE of three separate experiments.

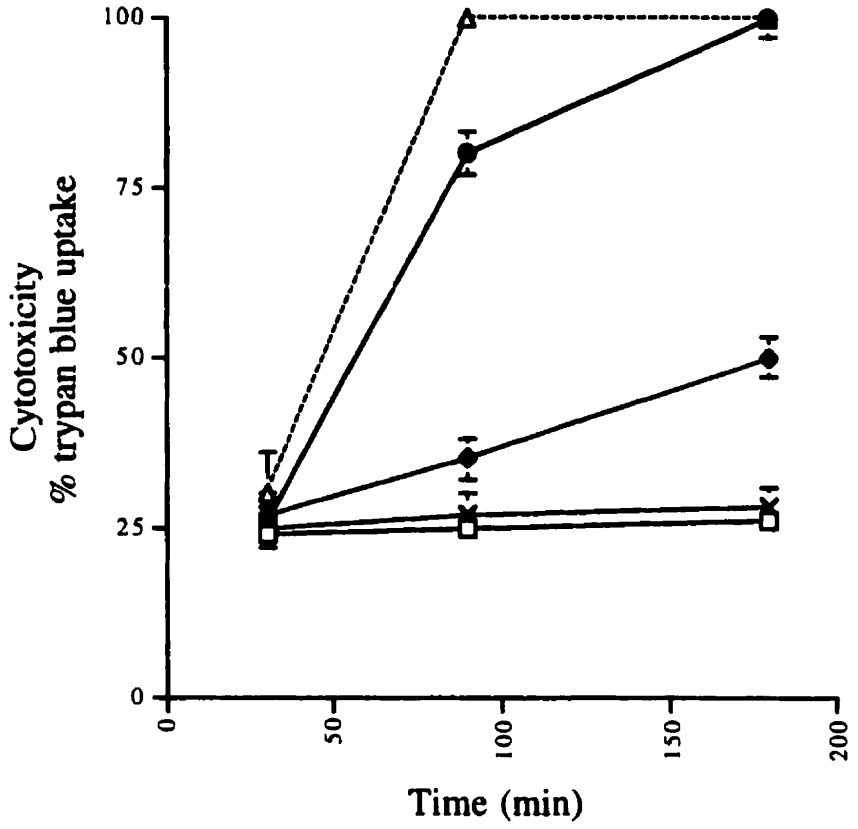


Figure 4.10: GSH depleted hepatocytes (with 1-bromoheptane) are more susceptible to Cu^{2+} and copper chelates than control hepatocytes. GSH-depleted hepatocytes (1 x 10^6 cells/mL) were incubated with Cu^{2+} (30μM) (◆), $Cu^{2+}(NC)_2$ (20μM) (Δ) and $Cu^{2+}(OP)_2$ (8μM) (●). Control hepatocytes (❑) and GSH depleted hepatocytes (X) with no additions. Results represent mean ± SE of three separate experiments.

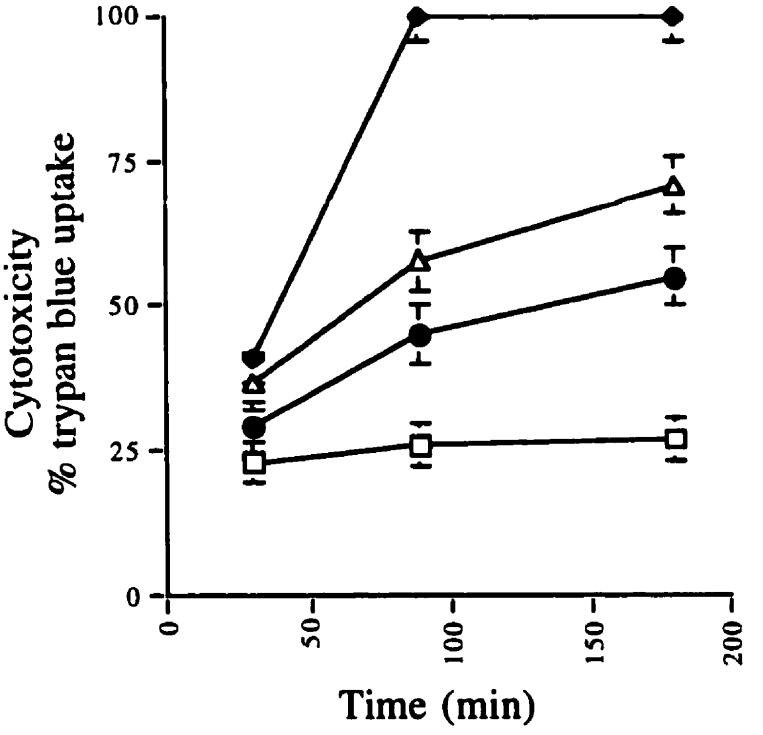


Figure 4.11a: The susceptibility of hepatocytes at 1% O_2 to Cu^{2+}, $Cu^{2+}(NC)_2$ and $Cu^{2+}(OP)_2$. Hepatocytes (1 x 10^6 cells/mL) were incubated with Cu^{2+} (30μM) (◆), $Cu^{2+}(NC)_2$ (20μM) (Δ) and $Cu^{2+}(OP)_2$ (8μM) (●). Control hepatocytes (❑) with no additions. Results represent mean ± SE of three separate experiments.

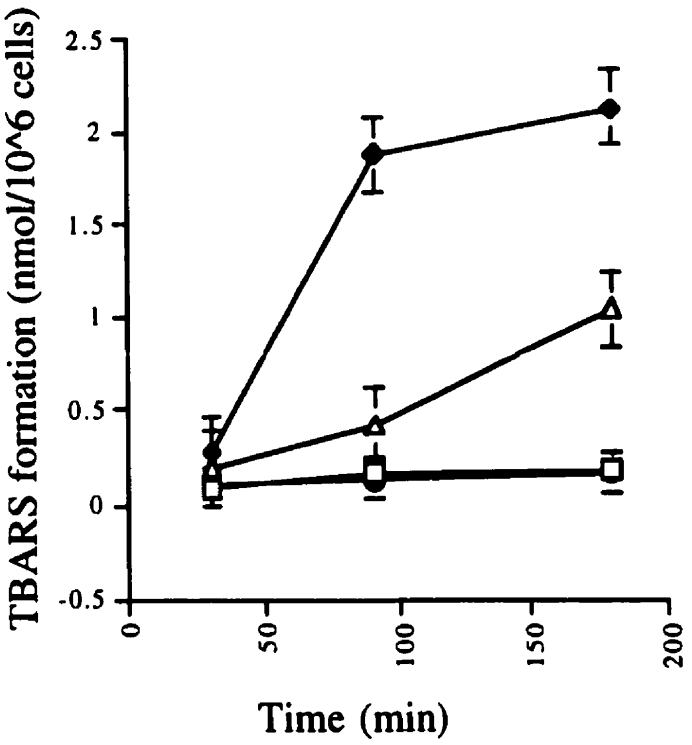


Figure 4.11b: Hepatocyte lipid peroxidation at 1% O_2 induced by Cu^{2+}, $Cu^{2+}(NC)_2$ and $Cu^{2+}(OP)_2$. Hepatocytes (1 x 10^6 cells/mL) were incubated with Cu^{2+} (30μM) (◆), $Cu^{2+}(NC)_2$ (20μM) (Δ) and $Cu^{2+}(OP)_2$ (8μM) (●). Control hepatocytes (❑) with no additions. Results represent mean ± SE of three separate experiments.

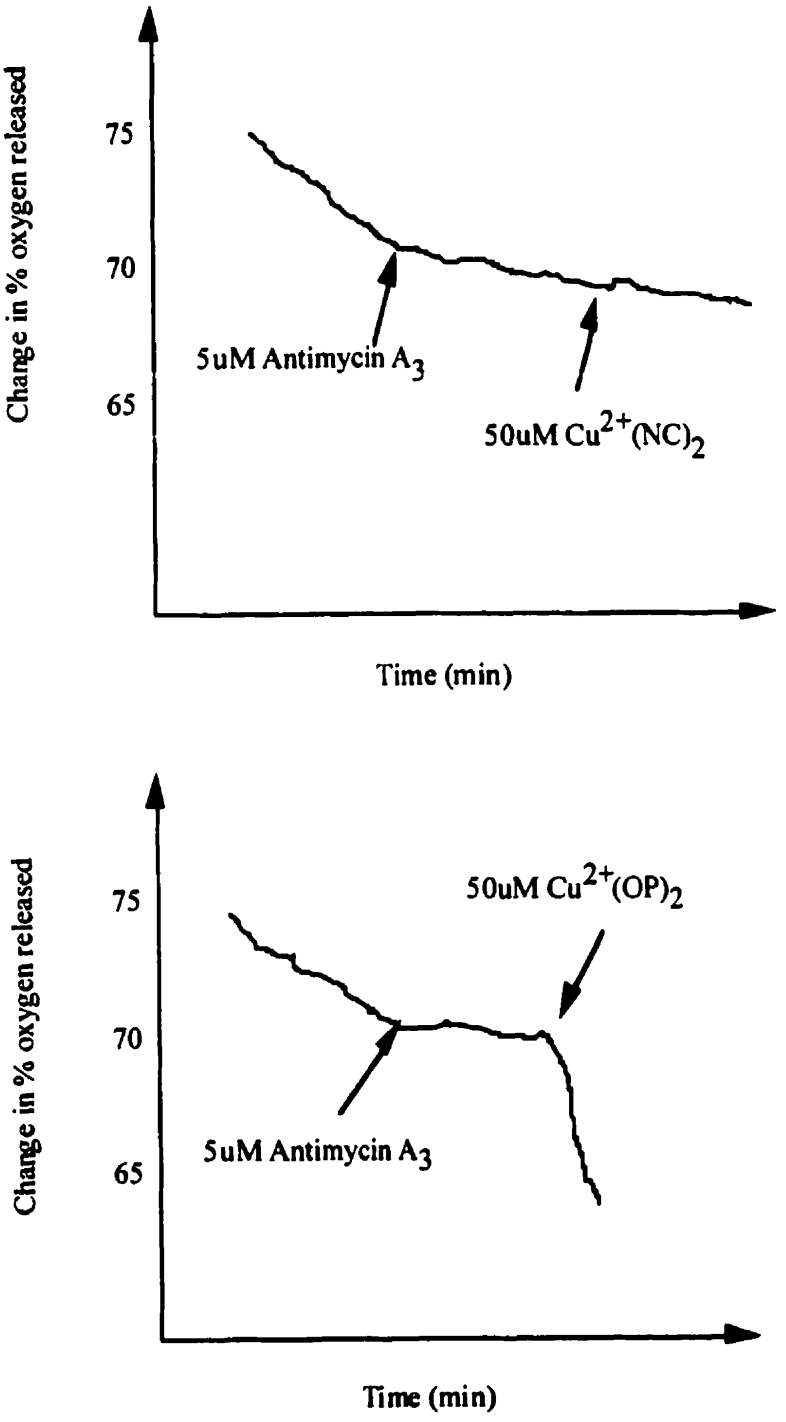


Figure 4.12: Antimycin resistant respiration induced by $Cu^{2+}(OP)_2$ but not $Cu^{2+}(NC)_2$. Additions were made to hepatocytes ($1x10^6$ cells). Results were obtained from 3 separate experiments in triplicate with freshly isolated hepatocytes

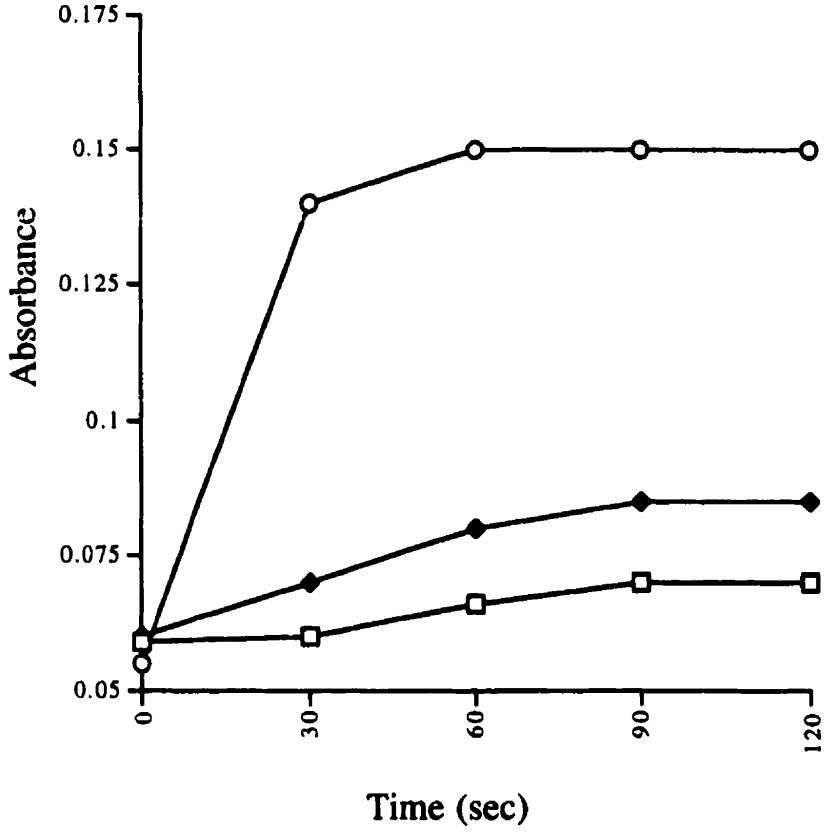


Figure 4.13: Hepatocyte reduction of $Cu^{2+}(NC)_2$ (50μM) at λ=454 nm and $Cu^{2+}(OP)_2$ (50μM) determined at λ=430 nm. The $Cu^{2+}(NC)_2$ reduction rate was enhanced in GSH depleted hepatocytes (○), but was slower in normal hepatocytes (◆). $Cu^{1+}(OP)_2$ (❑) formation was low as a result of rapid autoxidation.

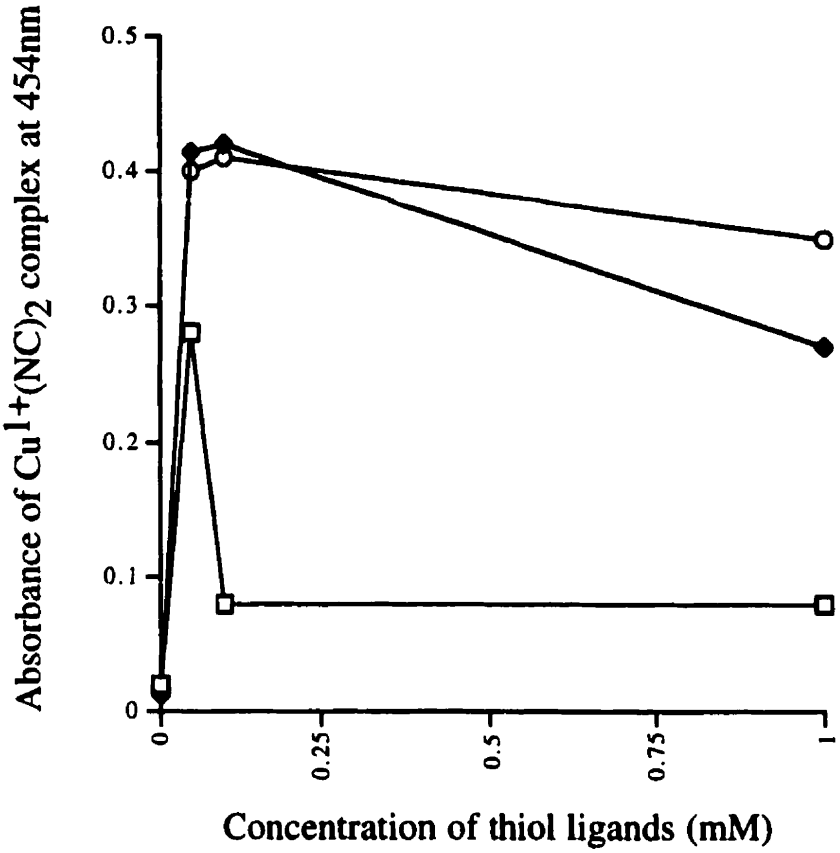


Figure 4.14: Reduction of $Cu^{2+}(NC)_2$ (50μM) by thiols at λ=454nm over 2mins at pH 7.4. Thiols were added in the concentration range of 50μM - 1mM as follows: DTT (❑), L-Cysteine (◆) and GSH (○).

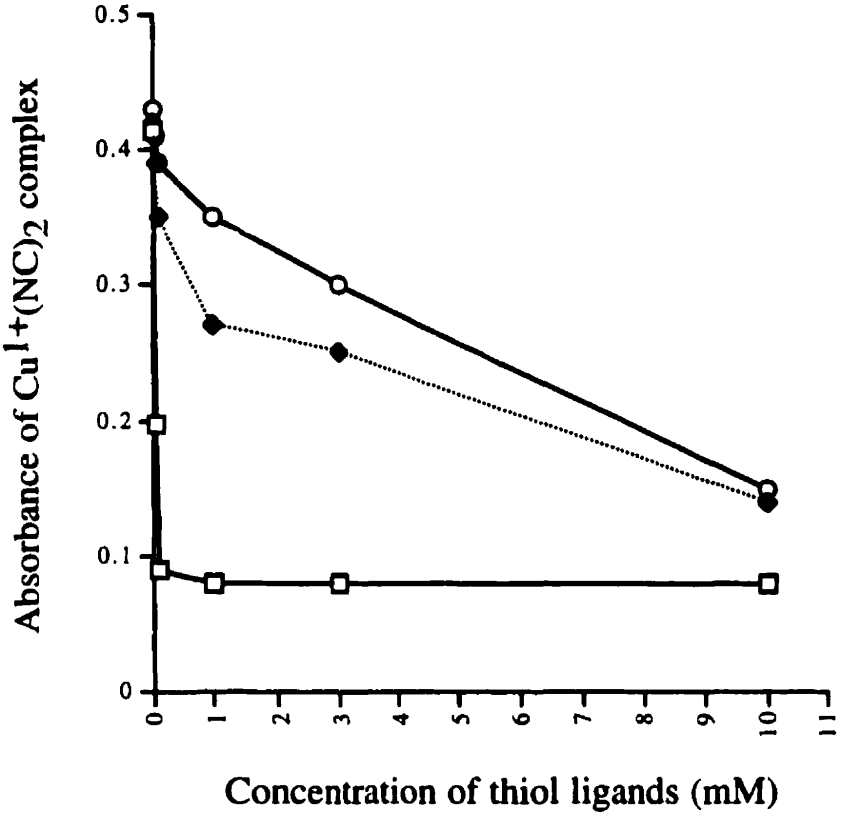


Figure 4.15: The interaction of $Cu^{1+}(NC)_2$ with thiol ligands . Time-based acquisition (2mins) was performed at λ=454nm (optimal for $Cu^{1+}(NC)_2$ complex) and pH 7.4 following the addition of the following thiols over a concentration range of 50μM - 10mM: DTT (❑), L-Cysteine (◆) and GSH (○).

4.3. DISCUSSION

In this present study, it has been demonstrated that $Cu^{2+}(OP)_2$ was 5-7 fold more effective than Cu^{2+} at causing cell lysis with freshly isolated hepatocytes. $Cu^{2+}(OP)_2$ toxicity was primarily O_2 dependent and resulted in significant amounts of lipid peroxidation. $Cu^{2+}(OP)_2$ cytotoxicity was not prevented by antioxidants, but was prevented by catalase, SOD, DMSO and the impermeable cuprous chelator, BCS. Other investigators have shown using atomic absorption spectroscopy that OP increases the uptake of Cu by Ehrlich ascite tumor cells. Furthermore, electron spin resonance spectroscopy of Ehrlich cells incubated with $Cu^{2+}(OP)_2$ revealed that $Cu^{2+}(His)_2$ was formed (Brynes et al., 1992). The cytotoxic mechanism of $Cu^{2+}(OP)_2$ toxicity therefore involves an increase in intracellular unchelated Cu^{2+} which undergoes intracellular redox cycling and oxygen activation resulting in nontoxic lipid peroxidation and cytotoxicity.

On the contrary, $Cu^{2+}(NC)_2$ cytotoxicity produced no detectable levels of lipid peroxidation. $Cu^{2+}(NC)_2$ toxicity was unaffected by BCS and catalase, but was partly protected by SOD. This suggests that $\bullet O_2^-$ contributes to $Cu^{2+}(NC)_2$ toxicity as it does with unchelated Cu(II). This observation is contrary to the physical chemistry of the $Cu^{2+}(NC)_2$ complex which is stabilized in the $Cu^{1+}(NC)_2$ form and does not react with O_2 to yield $\bullet O_2^-$ or with H_2O_2 to generate $\bullet OH$. This can be attributed to the high reduction potential of $Cu^{2+}(NC)_2$ as well as the high stability constant of the $Cu^{1+}(NC)_2$ complex (James et al., 1961; Goldstein and Czapski, 1985). An ESR analysis of Ehrlich cells

incubated with $Cu^{2+}(NC)_2$ showed that $Cu(His)_2$ was formed and rapidly lost (Brynes et al., 1992). In our studies, $Cu^{1+}(NC)_2$ formed in the hepatocytes transferred its Cu^{1+} to GSH as $Cu^{1+}(NC)_2$ rapidly disappeared on addition to normal hepatocytes but not when added to GSH-depleted hepatocytes.

Other investigators have shown that the uptake of NC by L1210 cultured cells was potentiated by copper and completely prevented if BCS was added to the culture medium. This suggests that a copper chelate-dependent uptake system in the cell membrane was responsible for transporting Cu^{2+} into the cell (Mohindru et al., 1983). In our studies, BCS failed to protect hepatocytes against $Cu^{2+}(NC)_2$ toxicity, indicating that Cu(II) was not released from the complex in the membrane as would be expected if Cu^{1+} uptake from $Cu^{2+}(NC)_2$ was mediated by a plasma membrane NADH oxidase (Berg and McArdle, 1994).

It has been hypothesized that the DNA strand breakage in Ehrlich ascites tumor cells induced by $Cu^{2+}(NC)_2$ more effectively than by $Cu^{2+}(OP)_2$ was caused ultimately by hydroxyl radicals formed from redox cycling of released unchelated Cu^{2+} (Brynes et al., 1992). However, $Cu^{2+}(NC)_2$ was much less effective than $Cu^{2+}(OP)_2$ in causing DNA oxidation when rat-liver nuclei were incubated with H_2O_2/ ascorbate (Burkitt et al., 1996). Thus to explain this apparent paradox, Brynes et al. (1992) suggested that $Cu^{2+}(NC)_2$ transfers its Cu to GSH by the following redox reactions:

$Cu^{2+}(NC)_2 + GS^- \Longrightarrow Cu^{1+}(NC)_2 + GS\bullet$ [1]

$Cu^{1+}(NC)_2 + GS^- \Longleftrightarrow 2NC + GS^- -- Cu(I)$ [2]

$GS^- -- Cu(I) + O_2 \Longrightarrow GS^- -- Cu(II) + \bullet O_2^-$ [3]

$GS^- -- Cu(II) \Longrightarrow GS\bullet + Cu(I)$ [4]

$2NC + Cu(I) \Longleftrightarrow Cu^{1+}(NC)_2$ [5]

$GS^- + Cu(I) \Longleftrightarrow GS\text{-}Cu(I)$ [6]

Nonetheless, from our results, it was observed that $Cu^{2+}(NC)_2$ was equally toxic at 1% O_2 or at 95% O_2. Similarly, GSH depleted hepatocytes were more susceptible to $Cu^{2+}(NC)_2$. Additional time-based spectral studies of $Cu^{1+}(NC)_2$ revealed that the complex was rapidly degraded by thiols in the following order: DTT >> L-Cysteine > GSH. It was thus proposed that DTT and cysteine rapidly removed Cu^{1+} from the $Cu^{1+}(NC)_2$ complex. The Cu^{1+}GSH complex formed in the hepatocyte is less cytotoxic than the alternative $Cu^{1+}(NC)_2$ or Cu(I) species as GSH depletion markedly increased the cytotoxicity of the $Cu^{2+}(NC)_2$ or Cu^{2+}. The two pathways concerning the fate of $Cu^{1+}(NC)_2$ could be distinguished as the initial addition of GSH followed by DTT to $Cu^{1+}(NC)_2$ failed to yield a significant reduction in levels of $Cu^{1+}(NC)_2$ whereas the reverse addition of reagents saw a rapid decrease in levels of $Cu^{1+}(NC)_2$ by DTT which was prevented by addition of excess GSH. DTT should therefore be highly cytoprotective as it could readily reduce and degrade the $Cu^{1+}(NC)_2$ complex based on its affinity for the Cu^{1+} ion.

It has been suggested that "free" copper released from the $Cu^{1+}(GSH)_2$ complex may participate in the sequence of reactions to yield •OH either in the cytosolic space or in the nucleus (Brynes et al., 1992). However, this seems unlikely as GSH-depleted hepatocytes were much more susceptible to $Cu^{2+}(OP)_2$ or $Cu^{2+}(NC)_2$. Furthermore, no intracellular GSH oxidation, lipid peroxidation or antimycin-resistant respiration was detected in the presence $Cu^{2+}(NC)_2$. Significant GSH oxidation obtained was prevented by cystine and was therefore a consequence of GSH leaking out of the hepatocyte (possibly due to membrane damage) before autoxidising in the medium. The •OH radical scavenger, DMSO or antioxidants also failed to prevent $Cu^{2+}(NC)_2$ cytotoxicity. This may be contributed by the $Cu^{1+}(NC)_2$ complex previously identified as a bidentate, tetrahedral structure (Hall et al., 1963) as opposed to the bidentate, distorted, planar $Cu^{2+}(NC)_2$ (Irving and Mellor, 1962), which may permeate the nucleus membrane and initiate site-specific damage on DNAby generating •OH. Yet, the scavenging effect of DMSO may be limited and nonspecific in cellular localization. Thus, it is most likely that the cytotoxic species was $Cu^{1+}(NC)_2$ or Cu^{1+} that can undergo redox cycling that damages cellular targets by forming reactive oxygen species.

At room temperature, Ehrlich cells incubated with $Cu^{2+}(OP)_2$ and $CuCl_2$ produced an ESR spectra similar to that shown by $Cu(His)_2$. It was postulated that the uptake of copper occurred by a similar pathway for $Cu^{2+}(OP)_2$ possibly as a $Cu(His)_2$ species (Brynes et al., 1992). The cytotoxicity of $Cu^{2+}(OP)_2$ was oxygen dependent and

caused significant lipid peroxidation under high oxygen levels, which became insignificant at low oxygen levels. Furthermore, the antioxidant DPPD did not prevent cytotoxicity. This suggested that lipid peroxidation is merely a side reaction. Both SOD and catalase provided protection, suggesting that $\bullet O_2^-$ and H_2O_2 are involved in the cytotoxic mechanism. BCS, an extracellular cuprous chelator also protected against $Cu^{2+}(OP)_2$ toxicity which could suggest that copper is released extracellularly from the complex prior to uptake by hepatocyte unlike the $Cu^{2+}(NC)_2$ uptake pathway. The OP ligand has been shown not to affect the uptake of copper and transport of both copper and the ligand occur by independent paths (Krishnamuri et al., 1980). Once Cu^{1+} enters the hepatocytes, it is redistributed possibly to available ligands, either GSH, free protein sulfhydryls or even OP and could account for the small amount of GSH depletion and GSH oxidation. Restoration of protein sulfhydryls by DTT protected against $Cu^{2+}(OP)_2$ cytotoxicity. $Cu^{2+}(OP)_2$ cytotoxicity was also increased in GSH depleted hepatocytes. It was also observed that NADH was a more effective reductant for $Cu^{2+}(OP)_2$ than GSH and L-cysteine (results not shown). Thus it is possible that $Cu^{2+}(OP)_2$ uptake involved the reduction of $Cu^{2+}(OP)_2$ to $Cu^{1+}(OP)_2$ prior to uptake by hepatocytes as the Cu^{1+} species. GSH may be required to ligate the free and reactive Cu^{1+} species, as GSH depleted hepatocytes were much more susceptible to $Cu^{2+}(OP)_2$ cytotoxicity. Clearly, much more experimental work is needed to distinguish how copper enters or is released in the hepatocyte when hepatocytes are incubated with $Cu^{2+}(OP)_2$ or $Cu^{2+}(NC)_2$.

In conclusion, we speculate that the $Cu^{2+}(OP)_2$ cytotoxic mechanism involves the reduction by the plasma membrane NADH oxidase resulting in the entry of Cu^{1+} into the hepatocyte. The Cu^{1+} is then chelated by intracellular ligands including GSH and protein thiols. Subsequent redox cycling of unchelated copper to generate $\bullet O_2^-$ and H_2O_2 in excessive amounts could cause oxidative stress cytotoxicity. The $Cu^{2+}(NC)_2$ cytotoxic mechanism on the other hand may involve the uptake of the copper complex across the plasma membrane. The intracellular $Cu^{2+}(NC)_2$ would be reduced and chelated by intracellular GSH, thiols or protein sulfhydryls. "Free" or unchelated Cu^{2+} could be reduced by various intracellular NADPH reductases. The "free" Cu^{1+} could cause oxygen activation at critical intracellular sites which results in cell lysis and are not accessible to DMSO. Figure 4.17 summarizes the cytotoxic mechanisms of both $Cu^{2+}(OP)_2$ and $Cu^{2+}(NC)_2$ in isolated hepatocytes and shows the role of chelators in determining the different effects on hepatocytes which may or may not result in cytotoxicity.

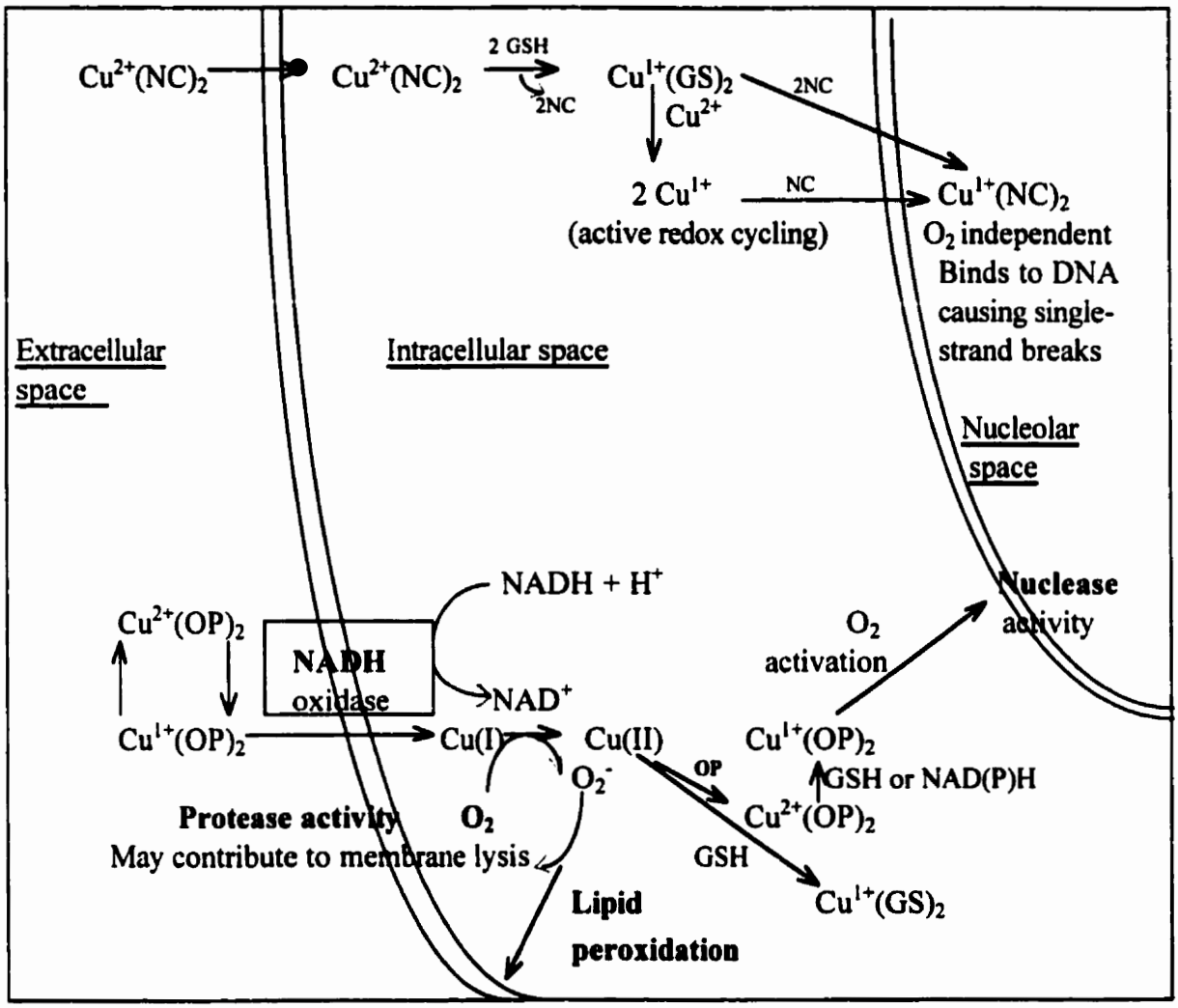


Figure 4.17: Schematic representation of proposed cytotoxic mechanisms of $Cu^{2+}(OP)_2$ vs. $Cu^{2+}(NC)_2$

Chapter 5

SUMMARY AND FUTURE PROSPECTIVE

5.1. SUMMARY

Ferric nitrilotriacetate ($Fe^{3+}(NTA)_2$) and cupric nitrilotriacetate (Cu^{2+} $(NTA)_2$) administration to mice and rats resulted in hepato and nephrotoxicity (Iqbal et al. (1995) and Toyokuni et al. (1992)) Since both transition metals are redox-active, it has been assumed that the cytotoxic pathways are similar. Nevertheless, the cytotoxic mechanisms of these metal complexes have not been clearly understood. It was the primary objective of this thesis to address the cytotoxic mechanisms of these two essential biological metals in the form of NTA complexes and identify their possible differences.

In **Chapter 3**, iron overload toxicity was attributed to lipid peroxidation and prevented by antioxidants. Copper overload toxicity, however, was mediated by reactive oxygen species and was prevented by ROS scavengers, namely catalase and SOD. Iron was also possibly involved in copper overload toxicity as the ferric chelator, desferoxamine prevented cell death. This also implies the toxicity of NTA is very much dependent on the nature of the bound metal. *In vivo* studies in CD1 mice treated with Cu^{2+} $(NTA)_2$ resulted in hepatotoxicity and nephrotoxicity after 24 hours. Both liver and kidney damage were prevented by desferoxamine, ammonium tetrathiomolybdate or leupeptin.

In **Chapter 4**, the cytotoxic mechanisms of the anticancer copper chelate complexes, $Cu^{2+}(OP)_2$ and $Cu^{2+}(NC)_2$ were compared. The $Cu^{1+}(OP)_2$ complex activates oxygen whereas $Cu^{1+}(NC)_2$ is inert. The results indicated that $Cu^{2+}(OP)_2$ cytotoxicity was oxygen dependent and induced significant lipid peroxidation. Cytotoxicity was prevented by ROS scavengers, BCS and DTT. Antioxidants prevented lipid peroxidation but failed to prevent cell death. It is likely that copper was released extracellularly, as BCS protected against cell death. $Cu^{2+}(NC)_2$ cytotoxicity also was prevented with SOD and DTT. Unlike $Cu^{2+}(OP)_2$, $Cu^{2+}(NC)_2$ did not cause significant lipid peroxidation or antimycin A_3-resistant respiration. However, $Cu^{2+}(NC)_2$ depleted cellular GSH at a rapid rate relative to copper or the $Cu^{2+}(OP)_2$ complex. In GSH-depleted hepatocytes, $Cu^{2+}(NC)_2$ cytotoxicity was greatly enhanced parallel with $Cu^{1+}(NC)_2$ formation. This suggests that Cu^{1+}-GS^- is not cytotoxic as has been assumed by other investigators.

5.2. Proposals for futher research

To substantiate our research, it is proposed that the following work will need to be carried out in order to elucidate the fate of the $Cu^{2+}(NC)_2$ and $Cu^{2+}(OP)_2$ complexes. Atomic absorption spectroscopy needs to be used to measure the uptake of copper by isolated hepatocytes. To confirm these results, experiments with radiolabelled $Cu^{2+}(NC)_2$ or $Cu^{2+}(OP)_2$ complex could be used to determine the uptake of copper by isolated hepatocytes. In addition, further work is required to clarify the proposed toxic role of

Cu^{1+}-GS^- intermediate and the role of GSH in copper distribution. This may involve ESR spectroscopy or more elaborate spectral analysis to determine the formation of physiological copper intermediates. The research can also be extended to studying the cytotoxic mechanisms of other copper complexes, such as thiosemicarbazone analogues which have also been identified as anticancer agents.

Furthermore, in our study of copper and iron overloading with respective NTA complexes, it was found that the toxicity of NTA complexes depended on the nature of the metal. Cu^{2+} $(NTA)_2$ was significantly more toxic than the $Fe^{3+}(NTA)_2$. It would be interesting to investigate the toxicity mechanisms of other essential biological metals namely calcium as NTA complexes. The toxicity of other important commercially or clinically used metal chelator can also be studied by chelation of iron or copper as the two most abundant transition metals in biological systems. Examples are pyridones and deferiprone (L1). Alternatively, the toxicities of copper and iron can also be studied as physiological complexes, in the form of citrate or histidine.

Finally, the possible phenomenon of iron involvement in copper overload toxicity should be further investigated by pretreating hepatocytes with desferal and determining the extent of toxicity upon overloading cells with copper. Measuring intracellular copper and iron levels with AAS may also identify if there is any release of iron from intracellular storage proteins induced by copper overload.

5.3. Future Application of Copper Complexes

In comparing the copper complexes, it was determined that $Cu^{2+}(NC)_2$ toxicity was dependent on cellular GSH levels. This suggested an important role of cellular GSH in detoxifying $Cu^{2+}(NC)_2$. Previous investigations with $Cu^{2+}(NC)_2$ have revealed that NC is a potent, copper-dependent cytotoxin with anti-tumor activity (Mohindru et al., 1983).

It may be feasible to enhance the anti-tumor activity of $Cu^{2+}(NC)_2$ specifically in liver and kidney tumors by depleting tumor GSH levels with buthonine sulfoximine without affecting tissue function.

To prevent copper overload toxicities, tetrathiomolybdate, desferoxamine and/or proteinase inhibitors may prove to be more effective and less problematical than the currently used penicillamine therapy.

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