Cardioprotective effects of dihydropyridine antagonists in a murine model of chronic iron-overload

by

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Abstract

Iron-overload cardiomyopathy is a leading cause of cardiovascular morbidity and mortality in the second and third decades of life worldwide, and occurs in patients with common genetic disorders such as hereditary hemochromatosis and beta-thalassemia major. Although the exact mechanism of iron-induced heart failure remains to be elucidated, the toxicity of iron in biological systems is hypothesized to be attributed to its ability to catalyze the generation of reactive oxygen species (ROS). There is preliminary evidence to suggest that non-transferrin-bound iron uptake in the myocardium may occur through L(long)-type calcium channels, and that calcium channel blockers (CCBs) may possess antioxidant properties. This thesis first examines the dose-dependent effects of chronic iron-loading on free radical mediated injury to lipid membranes, as quantified by the production of cytotoxic aldehydes. Secondly, it was hypothesized that the administration of two calcium channel blockers (nifedipine and amlodipine), would decrease iron-uptake in the myocardium, and decrease reactive oxygen species production in a murine model of iron-overload cardiomyopathy. The findings show that there are significant dose-dependent increases in heart tissue concentrations of iron, and ROS production in comparison to control mice in both models. Furthermore, the findings show that nifedipine is partially effective in limiting iron-uptake in the heart, but does not inhibit the production ROS in the heart of chronically iron-loaded mice. Investigations performed with amlodipine show that it also significantly inhibits the production of oxygen free radicals in chronically iron-loaded mice. These findings provide evidence for the first time of the possible iron inhibiting and antioxidant properties of L-type calcium channel blockers in an in-vivo model of chronic iron-overload.

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AMI	Acute myocardial infaction
Aml	Amlodipine besylate
Ca^{2+}	Calcium
CCB	Calcium channel blocker
CHD	Coronary heart disease
DDH_2O	Double distilled water
DHP	Dihydropyridine
Fe	Iron
GC-MS	Gas chromatography-mass spectrometry
H_2O_2	Hydrogen peroxide
HH	Hereditary hemochromatosis
HNE	4-hydroxynoneal
i.p.	Intraperitoneal
IRP	Iron regulatory protein
LDL	Low density lipoproteins
MDA	Malondialdehyde
mg	milligrams
mRNA	messenger radionucleic acid
Nif	Nifedipine
NTBI	Non-transferrin bound iron
OFR	Oxygen free radical

RE	Reticuloendothelial
ROS	Reactive oxygen species
SD	Standard deviation
TBA	Thiobarbituric acid

Chapter 1: Introduction and literature review

Iron-overload cardiomyopathy is an old disease that has evolved from a rare undiagnosable untreatable disease to a now much more common, diagnosable, and potentially preventable condition. Iron-overload cardiomyopathy manifests itself as a systolic or diastolic dysfunction secondary to increased deposition of iron in the myocardium (Hou, Wu, Lin, & Lue, 1994; Kremastinos, 1993). Pathologically however, it is due to a direct free iron effect on the myocytes, and not due to interstitial infiltration (Liu & Olivieri, 1994). This implies that the disease process is reversible if the tissue iron concentration can be controlled, such as through chelation therapy or pharmacological management. Despite its prominence, the mechanism of iron induced heart failure remains to be elucidated. One potential mechanism may involve the ability of low molecular weight iron to catalyze the generation of excess free radicals that can damage compounds of all biochemical classes (Lesnefsky, 1994; McCord, 1996). This thesis will examine the relationship between chronic iron–loading and free radical-mediated injury in the heart of mice as well as the antioxidant and iron-blocking effects of two separate L-type calcium channel blockers in the myocardium.

This chapter summarizes our current understanding between iron homeostasis, excess free radical generation, L-type calcium channel blockers and heart failure in disorders of iron overload. Specifically, this thesis will (a) discuss the role that iron may play in the generation of excess free radical species and their metabolites (e.g. aldehydes) and their potential association with cellular injury and ultimate myocardial dysfunction; (b) review the association between elevated concentrations of iron, alterations to antioxidant reserves and the development of cardiovascular disease including

cardiomyopathy; and finally (c) highlight the role of L-type calcium channel blockers may play in blocking iron-uptake in the heart and their antioxidant properties.

1.1 Iron metabolism

In solution, iron exists in two oxidative states, Fe (II) and Fe (III), which can donate or accept electrons respectively. At physiologic pH and oxygen tension, Fe (II) is readily oxidized to Fe (III), which rapidly forms essentially insoluble $Fe(OH)_3$ polymers (Ponka, 1999). Due to irons catalytic action in redox reactions, iron plays a key role in the formation of harmful reactive oxygen species (ROS) that can result in peroxidative damage to vital cellular components such as DNA, proteins, and mitochondria (Halliwell & Gutteridge, 1990; McCord, 1998). Specifically, oxidative damage is believed to be derived from the highly toxic hydroxyl radical (OH•) that is generated by the transition metal-catalyzed Haber-Weiss reaction, which will be discussed below in section 1.2.

Only 300 mg of iron is present at birth in humans, while the remainder accumulates over the life span to approximately 4 to 5 grams for males and 3 to 4 grams for females (Bodinski, 1987). Typically, adult men normally have 35 to 45 mg of iron per kilogram of body weight, while premenopausal women have lower iron stores as a result of recurrent blood loss through menstruation, which is approximately 30 mg of iron per menstrual cycle (Andrews, 1999). More than two-thirds of the body's iron is incorporated into hemoglobin in developing erythroid precursors and mature red cells. Each erythrocyte contains one billion atoms of iron and the erythron alone has a daily requirement of approximately 20 mg of iron, but only 1 to 2 mg of iron normally enters the body each day through the small intestine (Andrews, 1999).

1.1.1 Regulation of Iron Absorption

Actual iron derived from the diet is small yet significant because there is no known physiological mechanism for the excretion of excess body iron stores (Andrews, 1999). In the intestine, the duodenal crypt cells sense the iron requirement for the body and are programmed by that information as they mature into absorptive enterocytes. Enterocytes lining the absorptive villi close to the gastroduodenal junction are responsible for all iron absorption in the small intestine. Iron must pass from the gut lumen through the membranes of the erythrocyte to reach the plasma. Iron obtained from food is not bound to transferrin, and there is no role for transferrin within the lumen of the intestine. Instead, the low pH of gastric effluent helps dissolve ingested iron and provides a protein-rich milieu. This facilitates enzymatic reduction of ferric iron to its ferrous form by a brush-border ferrireductase (Riedel, Remus, Fitscher, & Stremmel,1995).

Heme iron is taken up by a separate process that is not well characterized and is the insoluble, nonprotein part of the hemoglobin (Kaplan, 2000). Inside the enterocytes, iron has two possible fates: it may be stored as ferritin, or it may be transferred across the membrane to reach the plasma. The determining factor is probably the "set point" that was established when the enterocyte developed from a crypt cell. Iron that remains in the form of ferritin as the enterocytes completes its life cycle will be sloughed with the senescent cell and will leave the body through the gastrointestinal tract (Andrews, 1999).

Iron absorption is regulated in several ways. The first mechanism is the dietary regulator, which refers to the amount of iron recently consumed in the diet. For several days after a dietary iron bolus, absorptive enterocytes are resistant to acquiring additional

iron (previously known as a mucosal block). The second regulatory mechanism responds to total body iron, rather than dietary iron. This mechanism has been termed the stores regulator, which is capable of changing the amount of iron absorbed to a limited extent (Finch, 1994). The final mechanism, known as the erythropoietic regulator, modulates iron absorption in response to the requirements for erythropoiesis (Finch, 1994). This probably involves a soluble signal that is carried by plasma from the bone marrow to the intestine.

1.1.2. Mechanisms of iron storage and transport

Ultimately, organisms had to develop a way to keep 'free iron' at the lowest level possible yet in concentrations adequate for the synthesis of hemeproteins and other iron-containing molecules. This has been achieved by the evolution of specialized molecules for the acquisition, transport, and storage of iron in a soluble, non-toxic form to meet the cellular iron requirements namely, transferrin and ferritin.

1.1.2.1. Transferrin

Transferrin functions to transport iron between sites of absorption, storage, and use. However, the mechanism and regulation of iron mobilization and transport from tissue stores to plasma transferrin are the least understood aspects of iron metabolism. Normally, plasma iron-concentration is approximately 18 μmol/litre, and total iron-binding capacity (a measure of plasma transferrin levels) is approximately 56 μmol/litre (Andrews, 2000). Thus, transferrin is saturated with only about one-third iron (approximately 30%) and the concentration of total plasma iron in the body

(approximately 3 mg) remains remarkably constant despite being turned over more than 10-times every day. Transferrin saturation is virtually unaffected by total body iron stores (ferritin and hemosiderin) that can vary from 350 to 900 mg in females and males, respectively (Bothwell, Charlton, Cook, & Finch, 1979). Hence, there is obviously a control mechanism in healthy individuals that ensures that the rate of iron released from body iron stores matches the one that the iron is taken up by tissues. However, in patients with severe iron overload disorders such as hereditary hemochromatosis and beta-thalassemia, plasma can contain transferrin that is completely saturated with iron. This results in the presence of non-transferrin bound iron (NTBI) in the circulation and is a pathological phenomenon thought to generally occur in patients with iron-overload disorders whereas NTBI is not detectable in healthy individuals (Breuer et al., 2000).

1.1.2.2. Ferritin and hemosiderin

Ferritin is a protein, which functions to sequester and store iron (Richardson & Ponka, 1997). Excess iron in the iron pool is bound to ferritin that can release ferric iron by undergoing pH-dependent proteolysis due to acid proteases in lysosomes (Ponka, 1999). When cellular iron concentrations rise, synthesis of transferrin receptors decreases while synthesis of ferritin increases. When the storage capacity of ferritin is exceeded, pathologic quantities of metabolically active iron are released intracellularly in the form of hemosiderin and free iron (Horowitz & Rosenthal, 1999). Mammalian ferritin consists of a protein shell that can accommodate up to 4500 atoms of iron in its internal cavity. Ferritin synthesis in inducible by iron by a mechanism in which iron recruits mRNA from

an inactive pool (Ponka, 1999). Hemosiderin is believed to be a degraded and polymerized form of ferritin, with a greater iron to protein ratio than ferritin.

1.1.2.3. Iron regulatory proteins

In mammals, iron homeostasis is partially regulated by two iron regulatory proteins (IRP-1 and IRP-2) (Cairo & Pietrangelo, 2000). These proteins display binding under conditions of iron deprivation, but become post-transcriptionally inactivated (IRP-1) or degraded (IRP-2) when the supply of iron to the cells is adequate (Hentze & Kuhn, 1996; Iwai et al., 1995, see Figure 1 below).

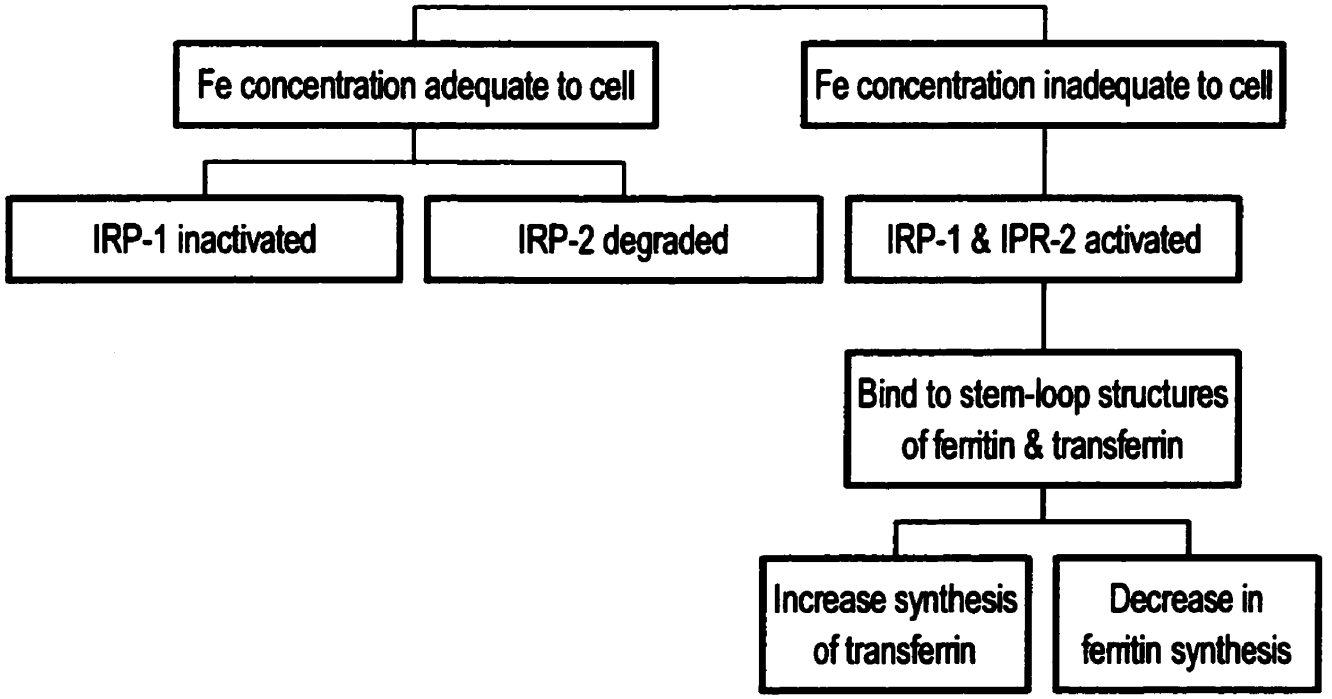

Figure 1: Role of IRP's in transferrin and ferritin. Schematic representation of IRP's. When Fe levels are low, IRP's are activated and bind to stem-loop structures of both ferritin and transferrin mRNA's. This binding stabilizes the transferrin mRNA and inhibits translation of the ferritin mRNA. Hence, when Fe levels are low there is an increase synthesis of transferrin and a decrease in ferritin synthesis. When there is an adequate supply of Fe, the opposite result is observed.

When chelatable iron levels are low, the IRPs are activated and bind to stem-loop structures of both ferritin and transferrin mRNAs (Meneghini, 1997; Haile et al., 1992). This binding stabilizes the transferrin mRNA and inhibits translation of the ferritin mRNA. Hence, when iron levels are low there is an increase synthesis of transferrin and a decrease in ferritin synthesis. Conversely, when there is an adequate supply of iron, IRP binding is deactivated and the opposite result is observed. IRP's have also been shown to be activated by nitric oxide, oxidative stress and the administration of hydrogen peroxide (H_2O_2) in experimental systems (Drapier, 1997; Pantopoulos et al., 1997; Tsuji et al., 2000). This is significant because exposure of mammalian cells to H_2O_2, a free radical species, has been shown to decrease synthesis of ferritin (Pantopoulos & Hentz, 1995).

1.1.2.4. Mitochondrial iron-uptake

A gene designated FRDA encodes a protein termed "frataxin" and has recently been linked to iron-uptake in the mitochondria and to the development of cardiomyopathy in Friedreich's Ataxia (Babcock et al., 1997; Kaplan, 2000). Friedreich's ataxia is a mitochondrial disease, believed to be attributed to increased oxidant damage resulting from excess mitochondrial iron. Under normal conditions, iron is used by the mitochondria to make iron-sulfer clusters and heme. Because iron is potentially toxic, its concentration in the mitochondria must be tightly controlled. Briefly, there is believed to be a decrease in mitochondrial iron export which results in a reduction of cytosolic iron. Decreased cytosolic iron has a feedback effect that results in increased expression of cell surface iron transporters (Kaplan, 2000). More transporters

increase the cellular uptake of iron, which again accumulates in the mitochondria. Essentially, the cell acts as if it were starved of iron while its mitochondria is overloaded with iron (Kaplan, 2000). More recent studies have demonstrated iron deposits in patient biopsy specifically in the mitochondrion-rich areas of the heart, though not within the mitochondria (Wong et al., 1999). Bartfay and others (1999) reported that in a murine model greater epicardial deposits of iron positive granules was observed, in comparison to endocardial deposits suggesting that deposition of iron appears to follow the blood circulation route. They also observed that iron loading could significantly alter and damage various cellular components including the mitochondria. Figure 2 summarizes the known proteins involved with iron metabolism in humans.

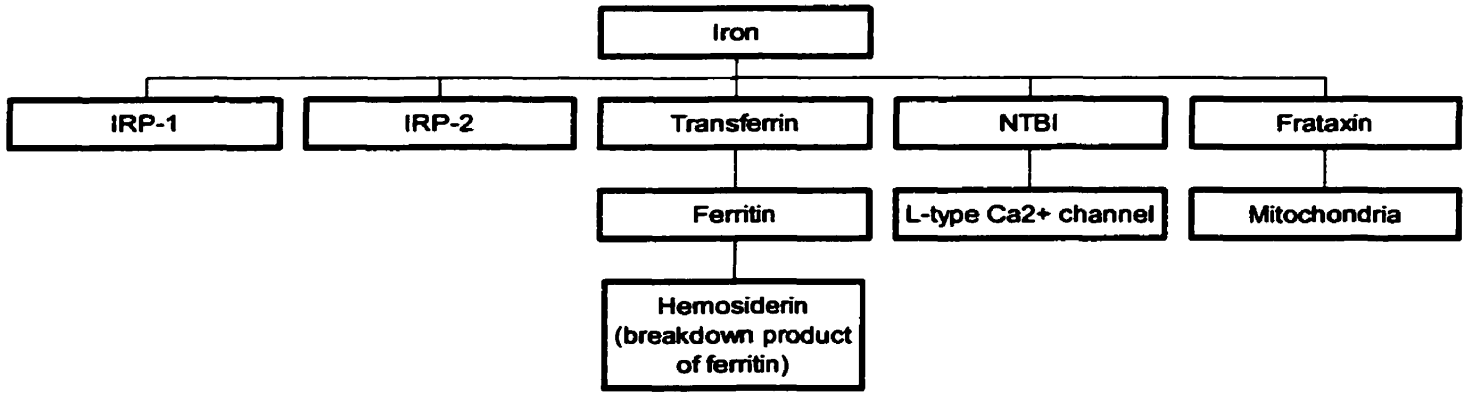

Figure 2: Summary of Iron Metabolism

1.2 Iron and Free Radical Generation

Free radicals are defined as a highly reactive chemical species that contain one or more unpaired electrons (Ball & Sole, 1998; Halliwell, 1997; Halliwell & Gutteridge, 1990). A compound can become a free radical either by gaining or losing an electron. The term oxidation refers to the loss of electrons by an atom or molecule, whereas reduction refers to the gain of electrons. The collective term "reactive oxygen species" (ROS) is used to describe both oxygen centered radicals (e.g., superoxide and hydroxyl radical) and also non-radical derivatives of oxygen such as hydrogen peroxide, singlet oxygen, hypochlorous acid (HOCl) and ozone (O_3) (Ball & Sole, 1998; Halliwell, 1997; Halliwell & Gutteridge, 1990).

In 1894, Fenton was the first to propose that the hydroxyl radical (OH•) could be generated in the presence of iron (Fe^{2+}) and hydrogen peroxide (H_2O_2).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\bullet$$

Four decades later Haber and Weiss (1934) proposed that the hydroxyl radical could also be generated by the reaction of the superoxide radical ($O_2^-\bullet$) with hydrogen peroxide.

$$O_2^-\bullet + H_2O_2 \rightarrow O_2 + OH^- + OH\bullet$$

The combination of reactions shown below are now commonly referred to as Fenton-type reactions and/or iron-catalyzed Haber-Weiss reactions (Halliwell & Gutteridge, 1990; McCord, 1996; Meneghini, 1997).

$$O_2^-\bullet + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\bullet$$

$$O_2^-\bullet + H_2O_2 \rightarrow O_2 + OH^- + OH\bullet$$

Kang and colleagues (1989) reported the formation of the hydroxyl radical in an experimental rat model of iron poisoning which consisted of intragastic instillation of ferrous sulfate. This investigation reported for the first time the presence of OH• and its dependence upon hydrogen peroxide during the oxidation of ferrous salts by molecular oxygen, confirming that the hydroxyl radical plays an important role in tissue damage after iron overload.

Oxygen free radical production in the biological system is a normal occurrence, which constantly generates free radicals species. For example, the immune system and in particular phagocytes, require free radicals to destroy bacteria and invading pathogens. When foreign organisms such as bacteria invade the body, the production of free radicals increases. Hence, the body must produce protective molecules in the form of antioxidants to protect the healthy cells from excessive ROS production (See figure 3).

Normal metabolic ROS Production

Normal Removal (antioxidants)

Figure 3: Normal metabolic ROS production. Figure 3 represents the concentration of ROS in biological system is kept in check by the antioxidant defense system.

However, for the Fenton reaction to occur at an excessive rate, iron in a reactive form is needed. Non-transferrin bound iron, ferritin, cytochromes and other iron containing enzymes have been proposed as potential sources of iron for catalyzing these reactions (Babbs, 1985; Ozaki, Kawabata & Awai, 1998; Voodg, Koster, vanEijk, & Sluiter, 1992).

Most iron in the body is bound safely in the form of hemoglobin or myoglobin. Cells are protected against iron-induced oxidative damage by specific iron binding proteins such as ferritin and transferrin (Halliwell, 1994; Kuehn, 1991). Under physiological conditions, these proteins protect against iron toxicity. To perform the role in the iron-catalyzed Haber-Weiss reaction, iron in most instances must be released from these proteins and chelated to low-molecular-weight forms such as iron citrate or iron ascorbate. Moreover, the superoxide ($O_2\bullet$-) radical produced during this reaction can

mobilize iron from ferritin (Abdalla, Campa, & Monterio, 1992; Halliwell, 1993, 1994), while H_2O_2 is capable of releasing iron from heme (deValk & Marx, 1999; Halliwell, 1994). Importantly, a potential source of catalytic free NTBI has been reported to be present in patients with iron-overload disorders (Al-Refaie et al., 1992; Grootveld et al., 1989)

The hydroxyl radical (OH•) is a highly reactive species second only to atomic oxygen in its oxidizing potential (McCord, 1996; Meneghini, 1997). Similarly, although the rate constant for Fenton-type reactions is higher for copper than for iron, the abundance of iron in biological systems makes it a more prominent source for hydroxyl radical generation (Lesnefsky, 1994; Meneghini, 1997; Sandstrom, Granstrom, & Marklund, 1994). Calculations based on rate constants suggest that hydroxyl radicals may be produced *in-vivo* at a rate of approximately 50 per cell per second (Halliwell, 1997). In fact, excess free radical production has been implicated in the pathogenesis of over 100 disorders, including diabetes, cancer and cardiovascular disease (McCord, 1996; Herbert et al, 1994; Halliwell & Gutteridge, 1990).

The mechanism of iron's toxicity is thought to be its ability to catalyze Fenton-type reactions when protective antioxidant defense systems (antioxidant reserves) become overwhelmed (Herbert et al, 1994; McCord, 1996; Sandstrom, Granstrom, & Marklund, 1994). These defense systems consist of both specialized enzymatic (e.g., glutathione peroxidase, catalase, superoxide dismutase) and non-enzymatic (e.g., vitamin E, selenium, β-carotene) biological molecules. For example, vitamin E (alpha-tocopherol) is an active and abundant chain breaking antioxidant present in lipid membranes (Gey et al., 1991; Kayden, & Traber, 1993; Rimm et al., 1993; Stampfer et

al., 1993). Gey and others have reported a significant inverse correlation between plasma vitamin E concentrations and mortality due to coronary heart disease (CHD). Two large scale prospective studies involving 39,910 men (Rimm et al., 1993) and 87,245 women (Stampfer et al., 1993) reported a 40% reduction in the incidence of CHD in individuals taking vitamin E supplements. Moreover, specific proteins (e.g., transferrin, ferritin) and chelators (e.g., deferoxamine, deferiprone), which bind transition metal catalysts, are also important defenses (Herbert et al, 1994; Ball & Sole, 1998). There is evidence to show that chronic iron-overload is associated with decreased concentrations of these protective antioxidants in experimental models of iron-overload (Bartfay et al., 1998, 1999), and in patients with primary and secondary hemochromatosis (Livrea et al, 1996).

1.3 Ischemia reperfusion: The role of Oxygen Free Radicals

Free radicals are generated during the reperfusion of an ischemic myocardium and the polyunsaturated fatty acids in the membrane phospholipids are the likely targets of ROS (Bagchi et al., 1997; Horwitz & Rosenthal, 1999). Notably, H_2O_2 and the superoxide radical ($\bullet O_2^-$) are generated during ischemia and reperfusion and appear in excessive concentrations and are capable of reducing ferritin-bound iron to the ferrous (Fe^{2+}) state, thereby further catalyzing the generation of the ROS (Babbs; McCord, 1985; Sandstrom, Granstrom, & Marklund; 1994).

For example, Chevion and workers (1993) measured the iron levels in the hearts of rats following reperfusion and reported that after the first coronary flow fraction (CFF) of reperfusion after 35 minutes of ischemia, the level of iron was 8- to 9-fold higher than the preischemic value. The levels of subsequent CFFs decreased and reached

preischemic values, indicating that iron appears in a burst at the resumption of coronary blood flow. These results are in accordance with the causative role that iron may play in heart injury following ischemia, by virtue of its capacity to catalyze the production of hydroxyl radicals.

Similarly, Cottin and colleagues (1998) reported that iron may promote the damage that occurs during ischemia and reperfusion, even in the absence of iron-overload by catalyzing the Haber-Weiss reaction. In their study they obtained peripheral venous blood samples from 17 men with acute myocardial infarctions (AMI) before thrombolytic therapy and after finding that iron increased significantly during reperfusion (mean time of peak was 9.4 +/- 7.3 hours) and returned to the pre-perfusion levels 48 hours post fibrinolytic treatment. They confirmed the importance of the temporal relationship between lipid peroxidation and iron status after thrombolytic therapy and concluded that they agree with the concept of using antioxidant agents in association with thrombolytic therapy.

It has been reported that intracellular calcium overloading and free radical generation are highly interrelated (Bagchi et al., 1997), thus suggesting a possible benefit for decreased calcium concentrations in the myocardium. Patients with iron-overload disorders may be particularly vulnerably to iron-catalyzed free radical generation if there is any component of ischemia, since the resultant intracellular acidosis will result in the release of massive amounts of "free" iron from ferritin and hemosiderin stores (Halliwell, 1989; Voodg et al., 1992; Ozaki, Kawabata, & Awai, 1988). Moreover, a significant plasma concentration of catalytic non-protein bound "free" iron has been reported in patients with iron-overload disorders and fully saturated transferrins, whereas none is

present in healthy controls (Al-Refaie et al., 1992; Breuer et al., 2000; Jakeman et al., 2001). In fact, there is preliminary evidence to show that patients with hereditary hemochromatosis (Gutteridge et al., 1985; Young et al., 1994) and beta-thalassemia major (Bartfay et al., 1999; Loebstein et al., 1998; Livrea et al., 1996) have increased free radical generation in plasma, as quantified by cytotoxic aldehyde-derived peroxidation products (e.g., hexanal, 4-hydroxynonenal, malondialdehyde).

1.4 Aldehydes as markers of free radical-mediated injury:

Aldehydes are relatively stable and biologically active breakdown products of lipid hydroperoxides formed by the reaction of free radicals with cellular membranes. Aldehydes are therefore employed as both markers and evidence for recent ROS-mediated activity and damage in vivo (Halliwell & Chirico, 1993; Luo et al., 1995). Lipid peroxidation in the biological system is always associated with the formation of aldehydes, which can vary in chain length depending on the site of oxidation and the location of the double bonds in the starting lipid (Grune et al., 1993; Requena et al., 1996). Unlike free radicals, aldehydes can diffuse and spread damage from their site of origin (Cheeseman, 1993; Luo et al., 1995), and may therefore act as secondary cytotoxic messengers for the primary free radicals generated (Yazdanpanah et al., 1997; Luo et al., 1995). A reaction that originally produces a single ROS that interacts within its own microenvironment may initiate a sequence of biochemical events that direct disturbances throughout the cell, its membrane and macroenvironment (Rice-Evans, 1990). Consequently, the presence of aldehydes is considered both markers and evidence for free radical formation in-vivo (Bartfay et al., 1999). Therefore, an increase in the

concentration of cytotoxic aldehydes in heart tissue of chronically iron-loaded mice implies a greater level of ROS production in the investigations detailed in this thesis.

Many assays are available to measure lipid peroxidation, but no single assay is an accurate measure of the whole process (Halliwell & Chirico, 1993). It has been reported that conversion of material into volatile derivatives separated by gas chromatography and identified by mass spectrometry (GC-MS) is likely to give more precise chemical information when complex mixtures are being studied (Thomas et al., 1991). Briefly, GC-MS measures lipid peroxides and aldehydes. Peroxidation products are extracted and reduced to alcohols, then separated by the GC, and identified by mass spectrometry (Halliwell & Chirico, 1993). Halliwell & Chirico 1993, reviewed various methods used to detect and measure biological lipid peroxidation products and have reported that GC-MS is the most effective test to measure peroxidative products in human material. They also stated that two other particular techniques known to measure lipid peroxidation products do not measure what they are supposed to measure: diene conjugation and the Thiobarbituric acid (TBA) test. Thomas and colleagues (1991) reported on the advantages of GC-MS which includes the sensitivity of the GC-MS to measure lipid peroxidation in as little as 1mg of tissue. While Luo and coworkers (1995) expanded on this knowledge and reported that lipid peroxidation produces a great variety of stable, diffusible aldehydes including malondialdehyde (MDA), 4-hydroxynoneal (HNE), and hexanal. Generally these three aldehydes are the most toxic and well characterized of the more than 20 known aldehydes. It is for this reason that the aldehyde results for MDA, HNE, and hexanal were chosen for the investigations.

1.5 Disorders of Iron Metabolism:

Iron-overload usually presents in one of two characteristic patterns. In cases with normal erythropoiesis (e.g. hereditary hemochromatosis), but where the plasma iron content exceeds the iron-binding capacity of transferrin, iron is deposited into the parenchymal cells of the liver, heart, and a subgroup of endocrine tissues (Andrews, 1999). In contrast, when iron overload results from increased catabolism of erythrocytes (e.g. β-thalassemia major), iron accumulates in reticuloendothelial macrophages first and then spill over into parenchymal cells. If left untreated, both forms of iron overload can progress to organ damage, dysfunction, and failure (Andrews, 1999).

1.5.1. Hereditary (Primary or Idiopathic) Hemochromatosis

Hereditary hemochromatosis (HH) is an autosomal recessive disorder caused by physiologically excessive iron absorption in the small intestines, which results in increased iron deposition in various organs (Bothwell & MacPhail, 1998). Typically, the erythroid cells are destroyed near the site of their development in the bone marrow and this process is termed ineffective erythropoiesis. Patients with hemochromatosis regularly absorb two to three times as much dietary iron as healthy individuals. Most do not have symptoms until adulthood, although the saturation of serum transferrin is usually increased by adolescence (Andrews, 1999).

Hereditary hemochromatosis is, in fact, one of the most common inherited genetic disorders in the world occurring with a homozygous expression of approximately 1 in

200 individuals of northern European descent (Horwitz & Rosenthal, 1999). Simon et al. (1976) discovered that the genetic predisposition for hemochromatosis originated with the HLA-A3 allele. Twenty years later the missense mutation C282Y was discovered to alter the class I-like protein designated HFE (Feder, et al., 1996). HFE forms a heterodimer with $beta_2$-microglobulin that is expressed on the surface of many cells, including duodenal crypt cells and macrophages. The C282Y mutation alters the conformation of the HFE protein and interferes with its function and has been shown to form a high-affinity complex with transferrin receptors (Andrews, 1991).

Interestingly, the homozygous expression does not always result in clinical disease, and heterozygous inheritance of only a single defective gene may result in increased iron stores, but does not usually result in clinical manifestations of the disease. On the basis of data from blood donors, it is estimated that as many as 1 in 10 white Americans carries at least one allele with this mutation (Edwards et al., 1988). Studies also show that symptomatic hemochromatosis has been estimated to occur in 1 in 500 individuals (Edwards et al., 1988). Clinically significant iron-overload typically develops in patients who are homozygous for the expression of this disorder. The diagnosis can be confirmed by direct mutation analysis of the HFE gene. Homozygosity for the C282Y mutation plus biochemical evidence of iron overload makes the diagnosis of hemochromatosis indisputable.

Heart failure and hepatic carcinoma remain the leading causes of death in this disorder with approximately one-third of patients dying of cardiac complications (Aldouri, Wonke, & Hoffbrand, 1990). Hemochromatosis often results in a restrictive cardiomyopathy, which is usually caused by an infiltrative disease of the myocardium.

Ultimately the myocardium becomes rigid and noncompliant, impeding ventricular filling and raising filling pressures during diastole. There is currently no therapy for restrictive cardiomyopathy other than treating the underlying disease process with phlebotomy (Barton et al., 1998). Treatment of HH has not changed substantially since 1950. Each 450 to 500 mL of blood contains between 200 to 250 mg of iron. Ideally, therapy should begin before symptoms develop, when the serum ferritin level exceeds 200 μg per liter in non-pregnant, premenopausal women or 300 μg per liter in men and postmenopausal women (Barton et al., 1998). Typically, phlebotomy is performed at a rate of 1 unit of blood per week until the patient has mild hypoferritinemia. On average, men require phlebotomy three to four times per year, while women require it one to two times per year (Barton et al., 1988)

There is preliminary evidence to show that patients with homozygous hereditary hemochromatosis have increased ROS production as quantified by aldehyde-derived peroxidation products in plasma (Sochaski et al., Young et al., 1994), and patients who have normal transferrin saturation have significantly more non-transferrin bound iron in their serum than normal control subjects (deValk et al., 2000). deValk et al (2000), investigated whether the serum of HH heterozygotes contained more NTBI than normal controls. By measuring the iron status of 27 treated homozygotes, 22 HH heterozygotes, and 17 healthy controls it was determined that NTBI was significantly higher in homozygous patients compared to heterozygotes patients ($p < 0.05$), and controls ($p < 0.05$). They concluded that the reported risk of heterozygous HH may be explained by NTBI-catalyzed LDL peroxidation.

Iron-overload is not limited to persons of Euopean descent. African iron-overload (formerly Bantu Siderosis) results from a predisposition to iron loading that is exacerbated by excessive intake of dietary iron. It is particularly problematic among Africans who drink traditional beer brewed in non-galvanized steel drums (Gordeuk et al., 1992) and may affect up to 10 percent of some rural populations (Gordeuk, 1992).

1.5.2. Secondary hemochromatosis (β-thalassemia)

The thalassemia syndromes represent the most common form of hereditary anemia in Canada and abroad (Lynch, 1995; Liu & Olivieri, 1994; Aldouri et al, 1994). It is estimated that approximately 100,000 children in the world are born annually with the disorder (Olivieri, 1999; UK Thalassaemia Society, 1993). Beta-thalassemia major is considered the prototypical secondary iron overload disorder characterized by chronic and severe anemia due to ineffective erythropoiesis and shortened red-cell survival (Dover & Valle, 1994). The severe ineffective erythropoiesis results in erythroid marrow expansion to as much as 30 times the normal level. Both an increase in plasma volume as a result of shunting through expanded marrow and progressive splenomegaly exacerbate anemia. Marrow hyperplasia leads ultimately to increased iron absorption and progressive deposition of iron in tissues (Olivieri, 1999).

The fundamental defect observed in the thalassemia syndromes is the uncoupling of alpha and beta chain synthesis. Nearly 200 different mutations have been described in patients with β-thalassemia that ultimately result in either the absence of the synthesis of β-globin chains or a reduction in synthesis. Both splicing of the mRNA precursor and ineffective cleavage of the mRNA transcript result in β-thalassemia. In some mutations,

no normal message is produced, whereas other mutations only slightly reduce the amount of normally spliced mRNA (Olivieri, 1999)

Figure 4 below is a schematic description of the metabolism of iron in thalassemia. Due to the increased rate of ineffective erythropoiesis, hemoglobin breakdown in reticuloendothelial (RE) cells results in the outpouring of catabolic iron and a 10-15 fold increase in plasma iron turnover. This results in complete saturation of transferrin and the emergence of non-transferrin bound iron (Hershko & Rachmilewitz, 1979; Hershko et al., 1978). The net increase in iron stores results from increased intestinal absorption and transfusions.

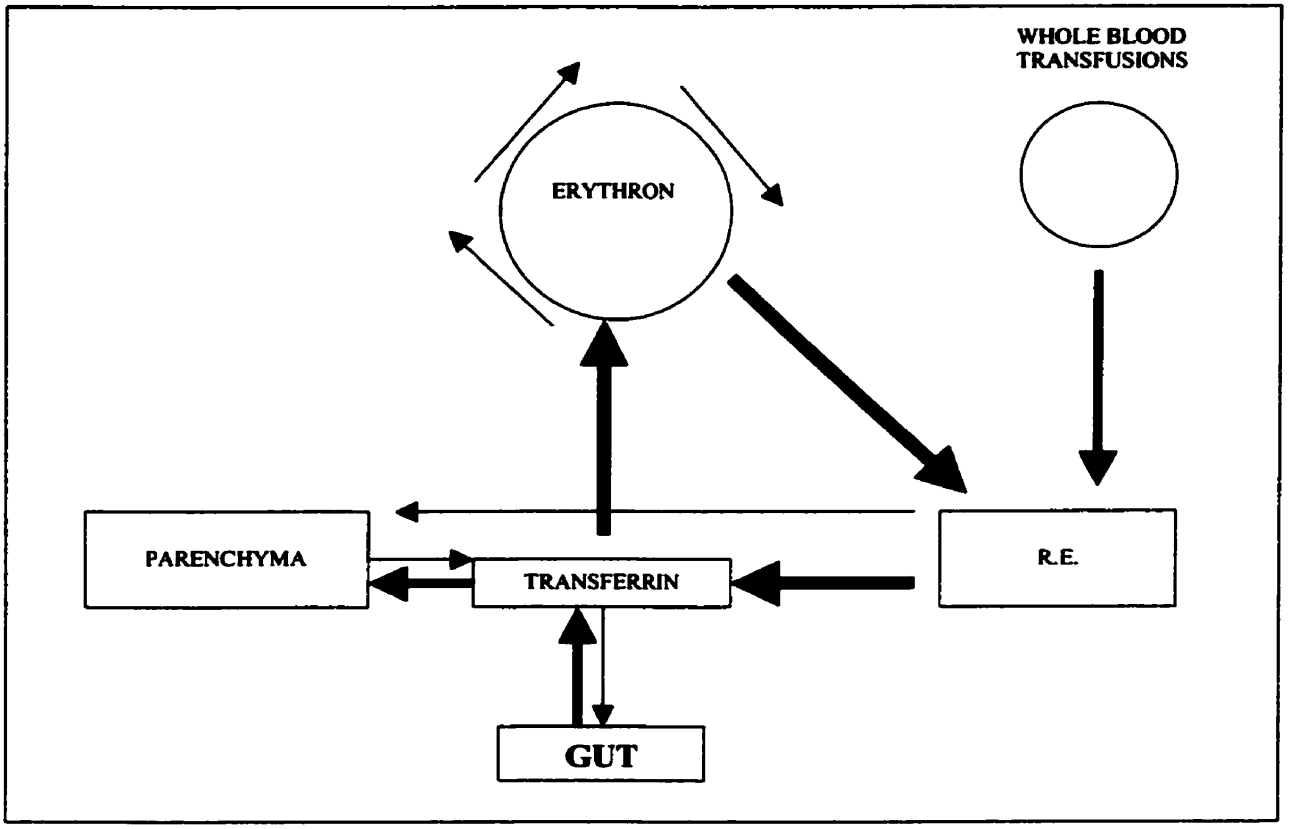

Figure 4: Iron Pathways in Thalassemia. Schematic representation of the metabolism of iron in thalassemia. Due to the increased rate of ineffective erythropoiesis, hemoglobin breakdown in RE cells results in the outpouring of catabolic iron and a 10-15 times increase in plasma iron turnover. This results in complete saturation of transferrin and the emergence of NTBI. The net increase in stores is caused by increased intestinal absorption and transfusions (adopted from Hershko et al., 1998 and Ponka, 1998).

Iron–overload of tissue, which is fatal with or without transfusion, is the most important complication of β-thalassemia (Olivieri & Brittenham, 1997). Although there are varying degrees of the disorder, most who have a homozygous expression become dependent of chronic blood transfusions to manage their underlying anemia (Horwitz & Rosenthal, 1999). As a result, large amounts of iron from transfused erythrocytes, hemolyzed red blood cells and dietary iron which are hyperabsorbed, accumulate and are incorporated in the heart as ferritin or hemosiderin.

Although most clinical manifestations of iron loading do not appear until the second decade of life in patients with inadequate chelation, evidence from serial liver biopsies in very young patients indicates that the deleterious effects of iron are initiated much earlier than this (Olivieri, 1999). Today, the prognosis for survival for patients without cardiac disease is excellent in those patients who receive regular transfusions of blood and whose serum ferritin levels remain below 2500 ng per millimeter with chelation therapy (Brittenham, Griffith, & Nienhuis, 1994). Bartfay and coworkers (1999) reported the positive effects of chelation therapy through decreased aldehyde formation in beta-thalassemia major patients who received standard chelation therapy with desferal (deferoxamine) or the experimental oral chelators L1 (deferiprone). Desferal was introduced 1979 and has increased life expectancy significantly to where some patients are now living into their forties (Liu & Olivieri, 1994). Nevertheless, these patients remain at risk for developing iron-induced organ failure because chelators remove only 10-20 mg of iron per day, and there is no know physiological mechanism for excreting excess iron from the body. The survival of patients with β-thalassemia is determined by the magnitude of iron loading within the heart (Brittenham et al., 1994).

Hence, chelation therapy is regarded as palliative and not curative in nature. Table 1 is adapted from Bottomley (1998) and is a summarization of the various forms of iron-overload.

Table 1: Disorders of Iron-overload

Disorder	Mechanism of Iron-overload
Hereditary (Primary) hemochromatosis	Recessive inheritance of increased Fe absorption
Iron-loading anemias:	Ineffective erythropoiesis, with or without red cell transfusions
Thalassemia syndromes	
Sideroblastic anemias	
Congentigential dyserythro-poietic anemias	
Excessive iron intake	
Red cell transfusions	Infusion of hemoglobin iron
Elemental iron	Prolonged ingestion of medicinal iron
Iron dextran	Prolonged parenteral iron therapy
African overload	Ingestion of excessive dietary iron plus a genetic factor(?)

1.6 L-type calcium channel blockers and free radical production:

Calcium antagonists are widely used for the treatment of patients with hypertension and cardiovascular disease (Resnic, 1998). Although the primary pharmacological role of calcium antagonists is to decrease vascular contractility by inhibition of the slow inward calcium ion channels, some of these antagonists have been reported to possess lipid antioxidant properties and free radical scavenging properties (Mak et al., 1995; Mason, Mak, Trumbore & Mason, 1999; Mason et al., 1999; & Yao et al., 2000). In fact, there is now preliminary evidence to demonstrate that ferrous (Fe^{2+}) uptake by the heart may occur through L-type calcium channels, which is the predominate type of calcium channel present in the heart (Digiesi et al., 2000; Farghali et al., 2000; Sevanian et al., 2000; Tsushima et al., 1999; & Winegar, Kelly, Larson, 1991).

Since the mechanism of non-transferrin bound iron (NTBI) uptake in the heart remains to be elucidated, it has also been proposed that NTBI may enter through L-type (long) calcium channels and contribute to the development of heart failure (Bartfay et al, 2000; Tsushima et al, 1999; Liu & Olivieri, 1994). Moreover, a common characteristic of all pathological conditions involving iron-overload is the observed concomitant calcium-overload in the heart (Anghileri & Thouvenot, 1998; Bagchi et al., 1997).

The L-type channel (dihydropyridine) is widely distributed throughout the body, particularly in the heart and smooth muscle cells (McMurray & Cleland, 2000). The high density of L-type Ca^{2+} channels in the heart has been shown through single patch-clamp recordings that Fe^{2+} is able to block Ca^{2+} currents and permeate these channels (Tsushima et al., 1999; & Winegar, Kelly, Larson, 1991). Tsushima and colleagues (1999) examined the ability of L-type Ca^{2+} channel modifiers to alter Fe^{2+} uptake by isolated rat

hearts and ventricular myocytes concluding that a component of Fe^{2+} uptake into the heart occurs via the L-type Ca^{2+} channel in an electrically arrested, nonbeating heart. They questioned in their discussion whether it would be reasonable to ask whether iron uptake via the L-type Ca^{2+} channel could account for the iron levels observed clinically. Tsushima and others noted that patients with secondary hemochromatosis often have total serum iron levels of 20 to 61 μmol/L, with estimated NTBI of ≈ 1 to 20 μmol/L. Furthermore, under these conditions the amount of iron accumulation predicted to occur via the L-type Ca^{2+} channel in 10-15 years, would be 3 to 5 mg of iron per gram of heart, which compares favourably with the 2 to 8 mg of iron per gram of heart typically observed in these patients.

Excess iron has also been implicated in the disruption of intracellular Ca^{2+} homeostasis (Kim et al., 1995; Link et al., 1996; Parkes et al., 1993; Tsushima et al., 1999; & Winegar et al., 1991). Specifically, Parkes and coworkers (1993) demonstrated in cultures of rat myocardiocytes that the increased rates of uptake of NTBI may exacerbate iron loading of the heart and contribute to iron-mediated cardiotoxicity. They found that iron loading with low-molecular-weight Fe promoted a dose- and time-dependent increase in the rate of uptake of NTBI that was positively correlated ($r = 0.9$, $p < 0.005$) with cellular iron content. This also resulted in decreased contraction of myocytes by 60%.

Similarly, various toxic heavy metals (e.g., mercury, lead) have been shown to result in a sustained elevation of Ca^{2+} with resultant cell death (Britton et al., 1991; Orrenius et al., 1992; & Whanger, 1993). Hence, from a therapeutic point of view it would be exciting to see whether iron entry into the heart could be prevented by the

administration of L-type Ca^{2+} channel blockers. Figure 5 below represents a schematic diagram of a cardiac myocytes and the proposed role of the calcium channel in regards to iron uptake into the myocardium, which is based on the available evidence to date.

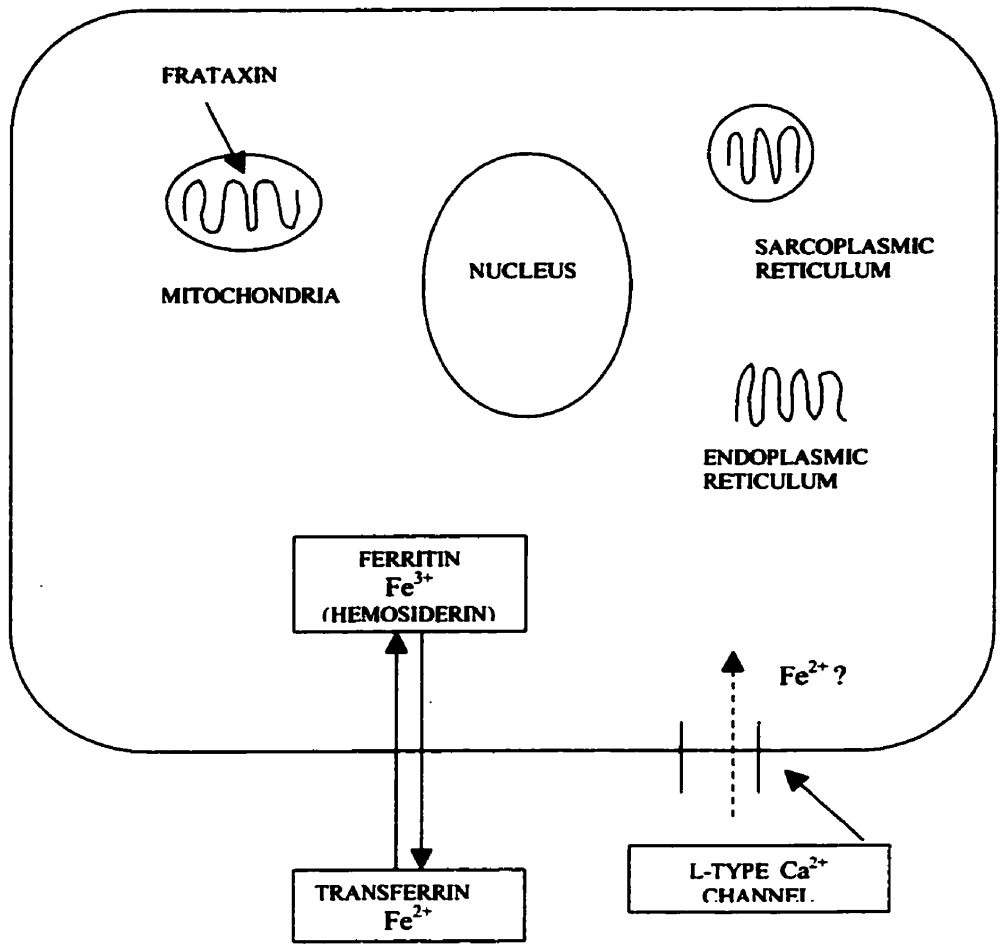

Figure 5: Simplified Model of a Myocyte Depicting Iron Transport and Storage. Schematic representation of a simplified model of a cardiac myocyte. The mitochondria is damaged by iron-overload, while the sarcoplasmic reticulum is dilated with Fe-overload. It is hypothesized that Fe^{2+} enters the myocyte through the L-type Calcium channel where ROS damage occurs.

NOTE: ⟶ Known

----▶ Suspected or hypothesized

1.6.1 Calcium channel blockers

Calcium channel blockers can be grouped by chemical class (benzothiazepines, phenyalkalamines, dihydropyridines or tetralol); or by their site of action (L 'long' or T 'transient'). L-type blocking agents reversibly bind distinct binding sites on the transmembrane spanning region of the L-channel. Binding is 'use dependent' meaning that other channels will not bind the drug in the open state.

The dihydropyridine (DPH) agents (such as nifedipine and amlodipine) have been the most extensively studied class of calcium channel blockers in patients with heart failure (McMurray & Cleland, 2000). The drugs in this group differ primarily in pharmacokinetics, which accounts for many of the striking differences found between the drugs. Two drugs classified within the dihydropyridine group, which are well characterized and used widely in the clinical setting, were chosen to be studied.

Nifedipine (Adalat, Bayer Canada) generally has a short elimination half-life in comparison to other members of this group, leading to a rapid onset and offset of action. However, the short duration of action of the DHP may result in reflex tachycardia that increases to some extent oxygen consumption (Nayler et al., 1987). It has recently been reported that nifedipine may possess beneficial antioxidant properties thus providing cytoprotective effects against ROS-mediated injury (Mak et al., 1995, Yao et al., 2000). Mak and colleagues (1995) reported that the cytoprotective effects of calcium channel blockers appear to be governed by antiperoxidative potency rather that calcium channel blocking activity. This was supported by Yao and colleagues (2000), who demonstrated that nifedipine had concentration dependent antioxidant effects. It is thought that nifedipine by virtue of its nitroaromatic structure may interact with the cascade of event

leading to peroxidation and alter the rate of oxygen consumption by the microsomal electron transport system (Engineer & Sridhar, 1989). In addition, nifedipine may also possess additional beneficial properties in modulating Fe-uptake in the heart via L-type calcium channels and may therefore be employed as an adjunct in chelation therapy with Desferal in conditions of iron-overload (Savigni & Morgan, 1996). If so, these properties may be of benefit for the clinical management of Fe-overload disorders. However, there have been conflicting reports with regards to the antioxidant properties of nifedipine. Janero et al. (1988) found that nifedipine had insignificant antioxidant properties compared with other DHP's. While similar results were found by Sugawara and coworkers (1996).

Amlodipine besylate (Norvasc, Pfizer Canada) in contrast is a very lipid soluble agent with a long elimination half-life of approximately 37 hours, and more favourable neurohormonal effects (Gaviraghi, Micheli, & Trist, 1995). The antioxidant activity of amlodipine appears to occur in a dose dependent manner as assessed by decreased concentration of malondialdehyde (MDA) in various experimental systems (Mason et al., 1999; Yao et al., 2000; Mason, Mak et al., 1999; Mason, Walter et al., 1999). In addition to its hemodynamic properties, amlodipine has been shown to inhibit cytokine-induce endothelial cell toxicity, independent of calcium channel modulation (Mason, Walter et al.,1999). Mason, Walter and colleagues (1999) reported that the antioxidant activity of amlodipine was at least 100-fold more potent than other DHP analogs. It is believed that the antioxidant effects may be attributed to the electron-rich aromatic ring that is characteristic of most "chain-breaking" antioxidants such as α-tocopherol (vitamin E). It has also been suggested that more lipophilic DHP's, such as amlodipine, may provide

more cytoprotective effects than other DHP's (Mak et al., 1995; Mason, Walter et al., 1999; Yao et al., 2000; Mason et al.,1999)

To my knowledge no studies to date have examined the in vivo effects of the L-type calcium channel blockers nifedipine or amlodipine in preventing iron uptake into the myocardium nor the antioxidant effects of these agents in an experimental model of iron overload.

1.7 Proposed conceptual model

Although the exact mechanism of iron's toxicity remains unclear, one potential mechanism may be the ability of NTBI to catalyze the generation of free radicals resulting in an increase in ROS production leading to an imbalance in the antioxidant system. Figure 6 is a proposed conceptual model of chronic iron-overload based on the currently known literature and empirical data to date linking chronic iron-overload to increased ROS production and ultimately increased morbidity and mortality.

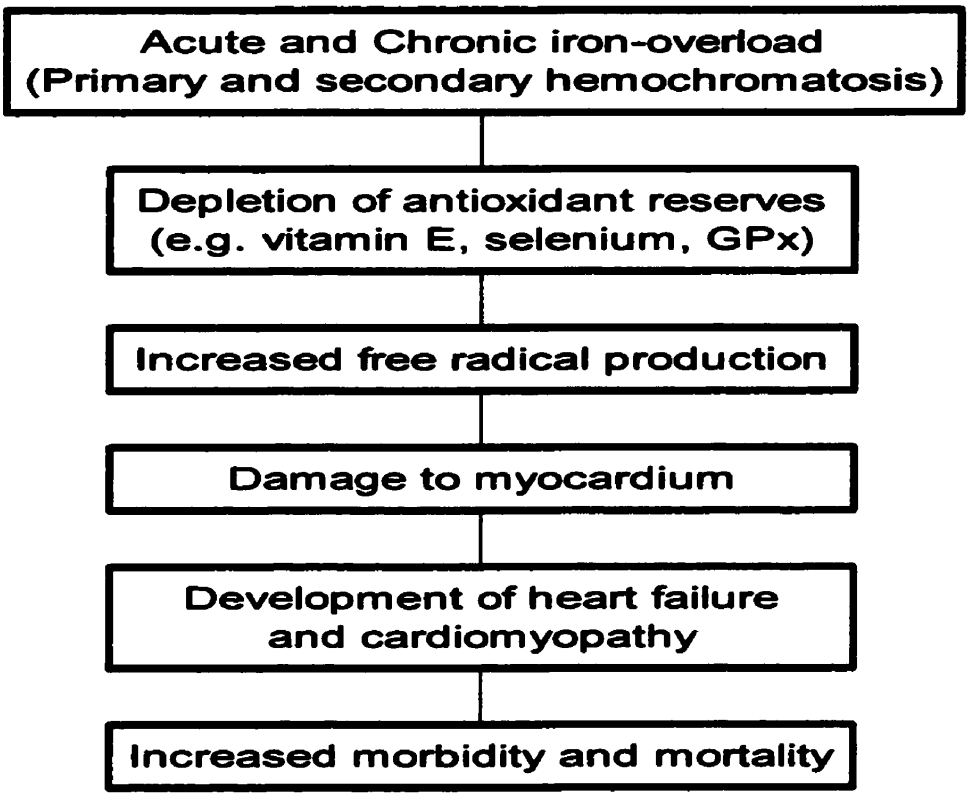

Figure 6: Proposed Conceptual Model of Chronic Iron-Overload. This is a conceptual model that proposes that patients with genetic disorders of iron metabolism (e.g. primary and secondary hemochromatosis) have decreased concentrations of protective antioxidants resulting in increased iron-catalyzed ROS generation. Excess ROS production damages the myocytes and various cellular components (e.g. mitochondria, lipid membranes), which ultimately leads to heart failure and the development of cardiomyopathy with increased morbidity and mortality.

1.7.1. Objectives

1. To determine if chronically iron-loaded mice will have dose-dependent increases in the concentrations of cytotoxic aldehydes (e.g., malondialdehyde, hexanal, 4-hydroxynonenal) in the heart, in comparison to vehicle control mice.
2. To determine if L-type calcium channel blockers prevent the uptake of iron in the chronically iron overloaded heart as quantified by tissue iron.

1.7.2. Hypotheses

1. Chronically iron-loaded mice will have increased total iron concentrations in the heart in comparison to control mice.
2. Chronically iron-loaded mice will have increased cytotoxic aldehydes present in the heart in comparison to vehicle controls.
3. Chronically iron-loaded mice that receive concurrent treatment with the L-type calcium channel blocker nifedipine will have decreased heart tissue concentrations of iron, decreased concentrations of cytotoxic aldehydes in comparison to iron-only treated mice.
4. Chronically iron-loaded mice that receive concurrent treatment with the L-type calcium channel blocker amlodipine will have decreased heart tissue concentrations of iron, and decreased concentrations of cytotoxic aldehydes in comparison to iron-only treated mice.

1.7.3. Overview of thesis

Chapter 2 describes the methods, materials, results, and discussion for Investigation I: Dose response study, which examines the dose-dependent effects of chronic iron loading. Chapters 3 and 4 examine the cardioprotective effects of Nifedipine and Amlodipine respectively, in terms of blocking iron uptake in the heart as well as their antioxidant properties with both chapters outlining the methods, materials, results and discussion for the specific investigation. Finally, Chapter 5 involves a critical discussion of the thesis and concludes with directions for further research.

2.0 Chapter 2

Investigation I: Dose response study

2.1 Aims and Rationale

Bartfay and colleagues (1999) previously examined the dose dependent effects of iron dextran of free radical production and heart tissue concentrations, but only in a pilot study. Hence, this study was replicated with a higher dose of iron dextran employed, as well as to determine the relationship between total dose of iron and concentration of aldehydes in the target area of the heart. The goal of this study was to determine if chronically iron loaded mice would have dose-dependent increases in the concentrations of cytotoxic aldehydes in the heart in comparison to vehicle controls. The specific hypotheses were (i) chronically iron loaded mice will have increased total iron concentrations in the myocardium, in comparison to control mice and (ii) chronically iron-loaded mice will have increased concentration of cytotoxic aldehydes in the heart, in comparison to vehicle controls.

Method

2.2 Sample

A standard murine model of iron-overload cardiomyopathy was employed for the experiments described below and pioneered by Bartfay and coworkers (1997). Male B6D2F1 mice (Charles River, Montreal) of 3 to 5 weeks of age (20 to 25 grams) were employed for all basic investigations reported in this thesis. These mice are produced by crossing C57BL/6 inbred female mice with DBA/2 inbred male mice and have genetic and phenotypic uniformity (Lewis et al., 1985; Liu et al., 1996; Skow et al., 1983). They have been used widely in research including transplant, radiation, behavioural, nutrient

and drug studies. Moreover, these mice are anemic and have increased gastro-intestinal absorption of iron closely mimicking similar biological conditions found in humans with iron-overload disorders.

2.3 Procedure

Chronic iron-overload was achieved by daily intraperitoneal (i.p.) injections iron dextran (Imferon), Sigma/Aldrich Chemical Company (St. Louis, MO), on five of seven days per week for a period of four weeks. It has been previously shown that parenteral administration of iron dextran unlike other models results in deposition of iron in the myocardium (Bartfay et al., 1997; Liu et al., 1996). Previous experimental models of chronic iron-overload that mimic the clinical situation have not existed (Liu et al., 1996). These previous models have entailed feeding the mice a diet high in carbonyl iron, which ultimately resulted in increased iron levels in the reticuloendothelial system in the liver, but not in the heart. It was reported that iron was present in the cardiac myocytes in a gerbil model of chronic iron-overload administered subcutaneously over a period of several months (Cathew et al., 1993). While, iron loading appears to result in greater epicardial deposits of iron in comparison to endocardial deposits in a murine model. This pattern of iron deposition appears to follow the blood circulation route of the myocardium from the epicardium to the endocardium, which is consistent with iron deposition patterns found in humans (Bartfay et al., 1999).

The mice were adapted to their surroundings for a period of three days before commencing treatments, and were given access to food pellets and drinking water ad libitum. The rodent diet (Laboratory Rodent Diet, PMI Nutrition International Inc., St.

Louis, MO) contained 184 parts per million of iron. The mice were housed in stainless steel cages (5 per cage) in a temperature- and humidity-controlled room with 12 hour light-dark cycles. This study had institutional approval (Queen's University Animal Care Committee) and confirms to the standards of the Animals for Research Act (Province of Ontario 1968-69, Rev. 1980) and the Canadian Council on Animal Care (Olfert and others, 1999). Unless otherwise stated, all chemicals used were reagent grade and were obtained from the Sigma-Aldrich Chemical Company (St. Louis, MO).

Iron loading was achieved by i.p. injections of iron dextran (20 mg per day per mouse), on five of seven days per week, for a duration of either one week (100 mg total iron dose, n=10), two weeks (200 mg total iron dose, n=10), or four weeks total (400 mg total iron dose, n=10). Control mice received placebo treatment for four weeks total with double distilled water (0.10 mL i.p. per mouse per day, n=10) [See Figure 7 below].

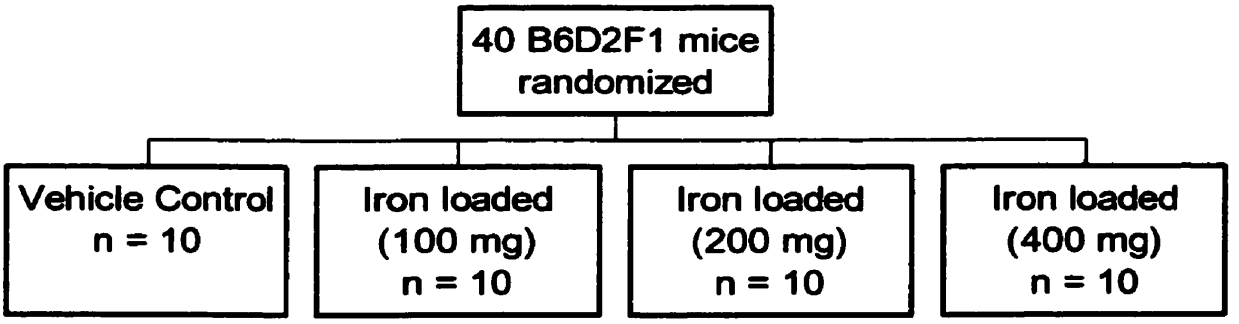

Figure 7: Dose Response Study

Mice were weighed in grams (± 0.1 g), on five of seven days before each injection. At the end of four weeks, the surviving mice were killed by cervical dislocation and the hearts were harvested via a rapid mid-sternal thoracotomy. The heart, spleen, and liver were harvested and the organs were subsequently cleaned of excess tissue, and rinsed in normal saline. Each organ was cut into two equal pieces and stored at –70 degrees Celsius until analysis for total tissue iron concentrations and oxygen free radical generation as detailed below in section 2.4 and 2.5. Mice that were deceased before the conclusion of the study were stored in a –70°C freezer until time of harvesting.

The following specific endpoints served as criteria to evaluate the objectives of this investigation: total concentration of iron, oxygen free radical production as quantified by the concentration of cytotoxic aldehydes in the heart (hexanal, HNE, MDA), and mortality rates.

2.4 Total Iron Concentrations

Calculations for total iron concentrations in heart tissue was performed at the Trace Metals Laboratories in London, ON by flameless atomic absorption spectrometry on an IL-55 Atomic Absorption / Atomic Emission Spectrophotometer (Instrumentation Laboratory, Wilmington, MA), as previously described (Bartfay and others, 1998). To ensure accuracy and precision, National Institute of Standards and Technology (NIST) traceable calibration standards were also employed (detection limit = 2 μcg per litre).

2.5 Oxygen Free Radical Production

Oxygen free radical production in heart tissue and plasma was quantified by the cytotoxic aldehydes hexanal, 4-hydroxynonenal (HNE), and malondialdehyde (MDA) by capillary column gas chromatography-negative ionization mass spectrometry (GC-MS) as previously described by Yazdanpanah and others (1997).

Briefly, sample preparation included 100 μL of plasma 10 μL of 10μ (100 pmol) of Benzaldehyde-ring-D_5 was added as an internal standard. The samples were vortexed for 30 seconds and to each sample 200 μL of 0.05M (10 μmol) of pentafluorobenzyl hydroxylamine hydrochloride was added to derivatize the carbonyl functional groups. The samples were incubated for 30 minutes at room temperature. The derivatized aldehydes were extracted twice with 1 mL of hexane. The extraction mixture was vortexed for 1 minute and centrifuged at 3000 rpm for 1 minute. The combined hexane extracts were evaporated under a stream of nitrogen, and 50 μL of trimethylsilyl + 1% trimethylchlorosilane for 5 minutes at 80°C to form TMS ether derivates of the hydroxyl functional groups. A 1 μL aliquot of the PFB-Oxime-TMS derivatives from each sample was then analyzed by GC-MS. The detection limit of this method is between 50 and 100 fenta-moles per 1 mL of injected aldehyde (Yazdanpanah and others 1997). This resulted in raw data for cytotoxic aldehydes expressed in pmoles for tissue samples.

2.6 Sample Size Estimates and Statistical Analysis

Based on statistical power analysis, it has been previously shown that 5 mice per treatment group is a sufficient number to detect differences between the treatment groups

with a power of .80 and an alpha level of 0.05 (Bartfay and Bartfay, 2000ab). Hence, justification was made for a minimum of 5 mice per treatment group and was employed for investigations I, II and III.

Descriptive statistics for the key endpoints (total iron, hexanal, HNE, MDA) are presented as mean ± standard deviation (S.D.) in a graphical format. A two-step procedure was employed for data analysis. One-way analysis of variance (ANOVA) was first performed to compare overall treatment effect and a p-value of less than 0.05 was deemed significant a priori. Second, when a statistically significant difference was detected, post-hoc multiple pair-wise comparisons were performed to determine the location and nature of the differences after ANOVA (See ANOVA table summary, Appendix A). Specifically, Duncan's Multiple Pair Wise Comparison was done and results are specified graphically.

2.7 Results

This investigation sought to determine if chronically iron loaded mice would have dose-dependent increases in the concentrations of cytotoxic aldehydes and iron in the heart in comparison to vehicle controls. The total amount of iron found within the heart appears to occur in a dose dependent manner as evidenced in Figure 8. The vehicle group had a total iron concentration of 344 μg/g of dry weight tissue. In comparison to this group, the 100 mg total dose of iron group had a 4.5 fold increase (1546 μg, $p < 0.05$) in total iron heart concentration. The groups that received 200 mg and 400 mg cumulative dose of iron had a 12.3 and a 19.9 fold increase in amounts of iron present within the heart respectively ($p < 0.05$ for all groups in comparison to control group).

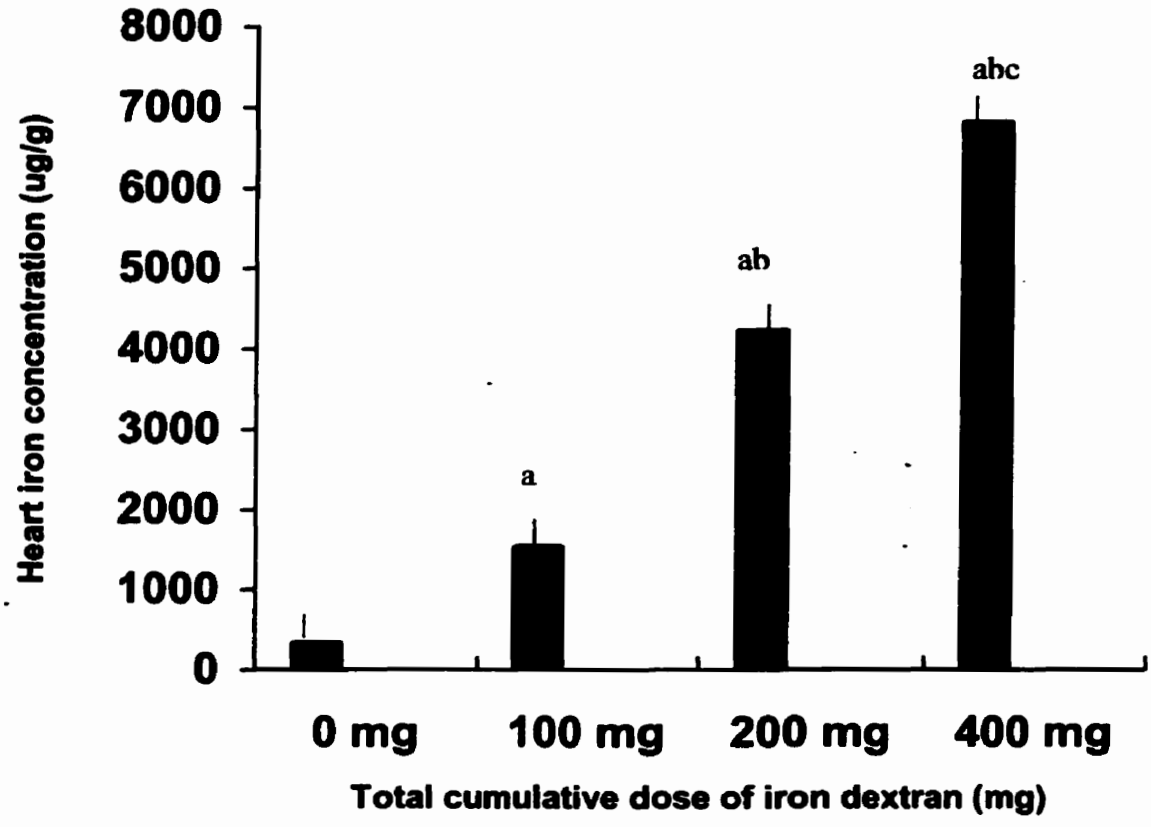

Figure 8: Heart Iron Concentration (ug/g dry weight tissue). Note the dose dependent increases in heart iron concentrations. All values are mean ± S.D., a = $p < 0.05$ compared to control, b = $p < 0.05$ compared to 100 mg group, and c = $p < 0.05$ compared to 200 mg group.

Heart tissue concentrations of the cytotoxic aldehyde hexanal are shown in Figure 9. In comparison to vehicle controls (154 pica moles {pmols} / 100 mg wet wt. tissue), a 1.3 fold increase (197 pmols, $p < 0.05$) in hexanal concentrations is observed in 100mg total iron dose treatment group, whereas a 1.6 fold increase (249 pmols, $p < 0.05$) and a 1.9 fold increase (298 pmols, $p < 0.05$) is noted in the 200 mg and 400 mg total dose of iron groups respectively.

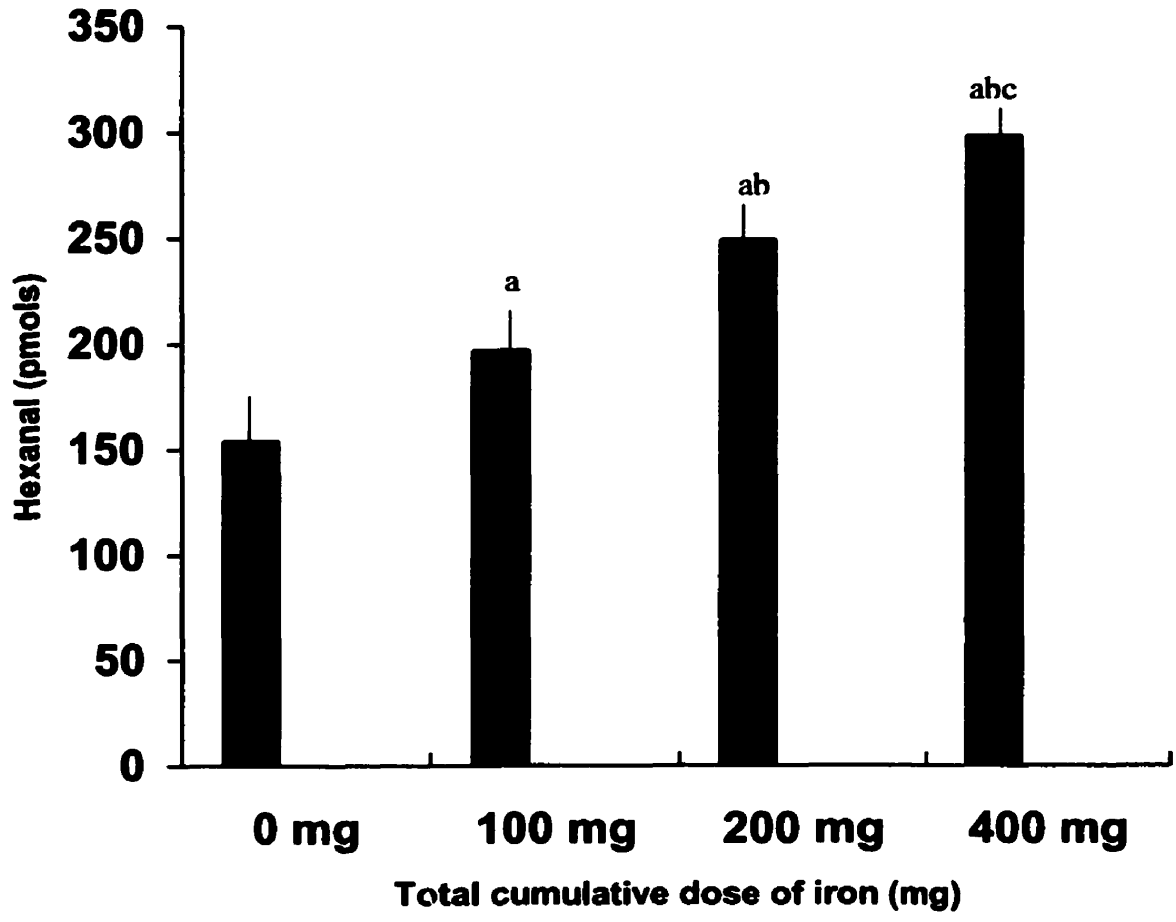

Figure 9: Heart hexanal concentrations (pmol/wet weight tissue). Note the dose dependent increases in hexanal production. All values are mean ± S.D., a = $p < 0.05$ compared to control, b = $p < 0.05$ compared to 100 mg group, and c = $p < 0.05$ compared to 200 mg group.

Heart tissue concentrations of the cytotoxic aldehyde 4-hydroxynoneal (HNE) are displayed in Figure 10. In comparison to vehicle controls (61 pmols), a 1.5 fold increase (90 pmols, $p < 0.05$) in HNE concentrations is noted in the 100mg total iron dose treatment group, whereas a 1.8 fold increase (112 pmols, $p < 0.05$) and a 3.0 fold increase (180 pmols, $p < 0.05$) is noted in the 200 mg and 400 mg total dose iron groups respectively.

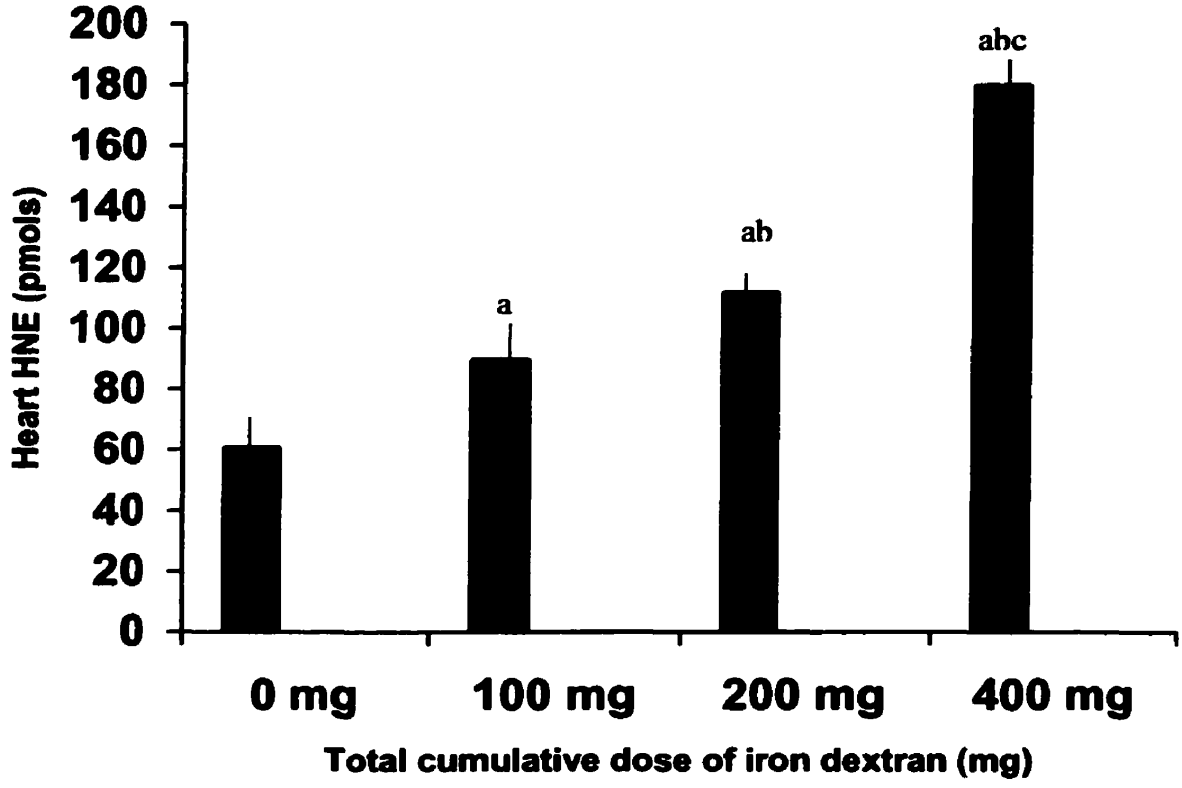

Figure 10: Heart HNE concentrations (pmol/wet weight tissue). Note the dose dependent increases in HNE production. All values are mean ± S.D., a = $p < 0.05$ compared to control, b = $p < 0.05$ compared to 100 mg group, and c = $p < 0.05$ compared to 200 mg group.

The heart tissue concentrations of the cytotoxic aldehyde malondialdehyde (MDA) are presented in Figure 11. In comparison to vehicle controls (1228 pmols), a 1.5 fold increase (1788 pmols, $p < 0.05$) in MDA concentrations is observed in the 100 mg total iron dose treatment group, whereas a 2.2 fold increase (2658 pmols, $p < 0.05$) and a 3.2 fold increase (3960 pmols, $p < 0.05$) is reported in the 200 mg and 400 mg total dose of iron groups respectively.

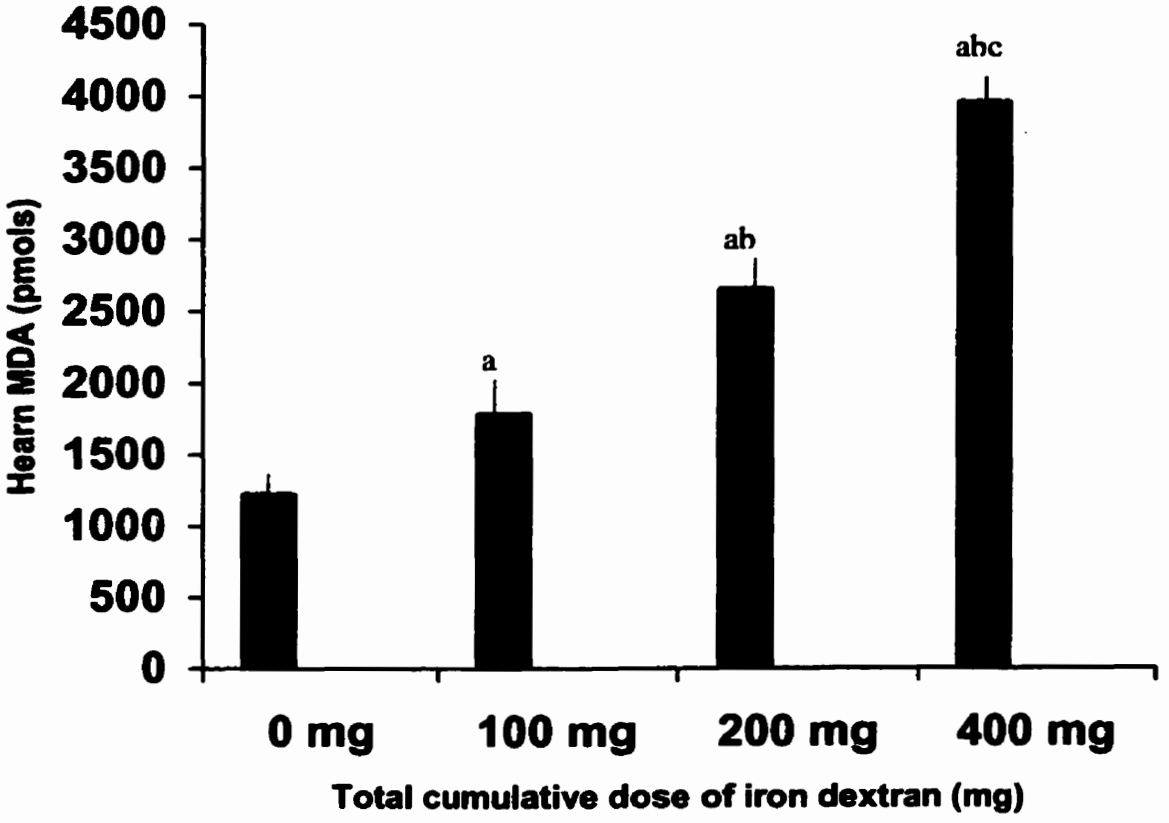

Figure 11: Heart MDA concentrations (pmol/wet weight tissue). Note the dose dependent increases in MDA production. All values are mean ± S.D., a = $p < 0.05$ compared to control, b = $p < 0.05$ compared to 100 mg group, and c = $p < 0.05$ compared to 200 mg group.

In conclusion, these results show that iron uptake occurs is a dose-dependent manner ($p < 0.05$) with marked progressive increases in Hexanal ($p < 0.05$), MDA ($p < 0.05$), and HNE ($p < 0.05$).

2.8 Discussion

It was shown that chronic iron loading in a murine model could result in dose-dependent increases in total heart iron concentration when iron dextran was administered intraperitoneally. To our knowledge, no studies to date have investigated the dose-dependent effects of chronic iron loading on heart tissue concentrations of iron, production of cytotoxic aldehydes, and ultimate mortality in this model concurrently. Accordingly, it was hypothesized that chronically iron loaded mice would have increased heart tissue concentrations of iron and aldehydes when compared to control mice.

HYPOTHESIS 1: Chronically iron-loaded mice will have increased total iron concentrations in the heart in comparison to control mice.
HYPOTHESIS 2: Chronically iron-loaded mice will have increased cytotoxic aldehydes present in the heart in comparison to vehicle controls.

In support of hypotheses 1 and 2, it was shown that chronic iron loading in mice results in significant dose-dependent increases in heart tissue concentrations of iron, increase in oxygen free radical production as quantified by various aldehydes and an increase in morbidity in comparison to non-iron loaded mice. This particular study was an extension of previous work done by Bartfay (1999) who performed a dose response study in a similar mouse model, but for a shorter period of time, and also employed lower dosages of iron dextran. This makes this particular investigation unique in that Canadian mice were used and purchased from Charles River, Montreal versus American mice purchased from Barharbor, Maine and found consistent results despite different suppliers.

Furthermore, this investigation was conducted for a longer period of time (4 weeks) than in previous investigations, and higher doses of iron dextran was administered with results from this study consistent with previous results. Based on the findings reviewed above, it is concluded that there is sufficient evidence to support the hypotheses set out for Investigation I.

3.0 Chapter 3

Investigation II: Nifedipine intervention study

3.1 Aims and Rationale

The heart has a high density of L-type Ca^{2+} channels, and single patch-clamp recordings have shown that Fe^{2+} is able to block Ca^{2+} currents and permeate these channels (Tsushima et al.,1999; Kim et al., 1995; Winegar et al, 1991). There is a growing body of evidence to demonstrate that calcium channel blockers may also possess antioxidant and free radical scavenging properties (Digiesi et al., 2000; Farghali et al., 2000; Sevanian et al., 2000; Sugawara et al., 1996). To our knowledge, however, no investigations to date have examined the iron inhibiting nor antioxidant properties of the L-type calcium channel blocker (CCB) nifedipine in an experimental model of iron-overload cardiomyopathy (Crowe & Bartfay, 2001ab). The specific hypotheses for this experiment were (i) chronically iron-loaded mice that receive concurrent treatment with nifedipine will have decreased heart tissue concentrations of iron in comparison to iron-only treated mice and (ii) chronically iron-loaded mice that receive concurrent treatment with nifedipine will have decreased concentrations of cytotoxic aldehydes in comparison to iron-only treated mice.

Method

3.2 Sample

The sample population was the same as detailed in Investigation I: Dose response study (see 2.2, p. 35).

3.3 Procedure

Iron loading was achieved by 20 mg (0.2 mL) i.p. injections of iron dextran. Each group consisted of 5 mice per group for a total of three groups. The first group was the control group and received 0.2 mL of double distilled water i.p. per mouse per day for a period of four weeks. The second group was the iron only group and received 20 mg of iron dextran i.p. per mouse per day. The final group was the iron plus nifedipine group. In this group, 0.2 grams of Nifedipine was crushed and mixed into 200 mL of drinking water along with 3.8 grams of sucrose for palatability. The solution was wrapped in tin foil to eliminate decomposition of the nifedipine due to light exposure and subsequently vortexed for 2-3 minutes to allow the solutes to dissolve. This mixture was changed twice a week (Monday and Wednesday) with no treatment occurring on weekends [see Figure 12 below]. The rest of the study remained the same, including specific endpoints, as described above for Investigation I: Dose response study.

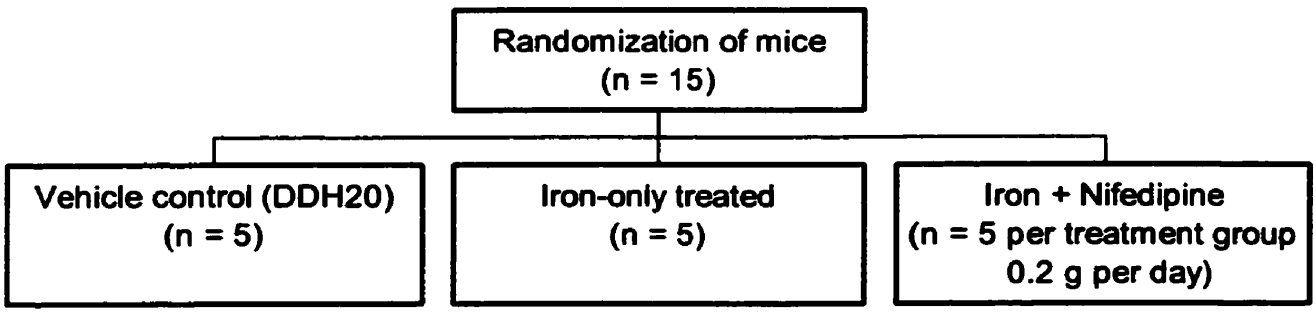

Figure 12: Nifedipine Intervention Study

3.4 Results

This investigation sought to determine the relationships between the total dose of iron administered and the iron inhibiting as well as the antioxidant properties of nifedipine in a murine model. Consistent with previous findings, we observed that the mice became progressively lethargic and inactive with increasing total iron burden (Crowe & Bartfay, 2001; Bartfay & Bartfay, 2000a, 2000b; Bartfay et al., 1998). The chronically iron-loaded mice when compared to DDH_2O treated mice had coarse fur with loss of colour and their major internal organs (liver, spleen, heart) appeared bronze in colour, when compared to the normal pink coloured organs in vehicle controls.

Figure 13 shows heart tissue concentrations of iron with assigned treatments. In comparison to vehicle controls (150 μg), iron only treated mice (12678 μg) had a 84.5 fold increase ($p < 0.05$) in total iron concentrations, whereas iron and nifedipine treated mice (8642 μg) had a 57.6 fold increase ($p < 0.05$). In comparison to iron-only treated mice, iron-loaded mice that received supplementation with nifedipine had a 1.5 fold decrease ($p < 0.05$) in heart tissue concentrations of iron.

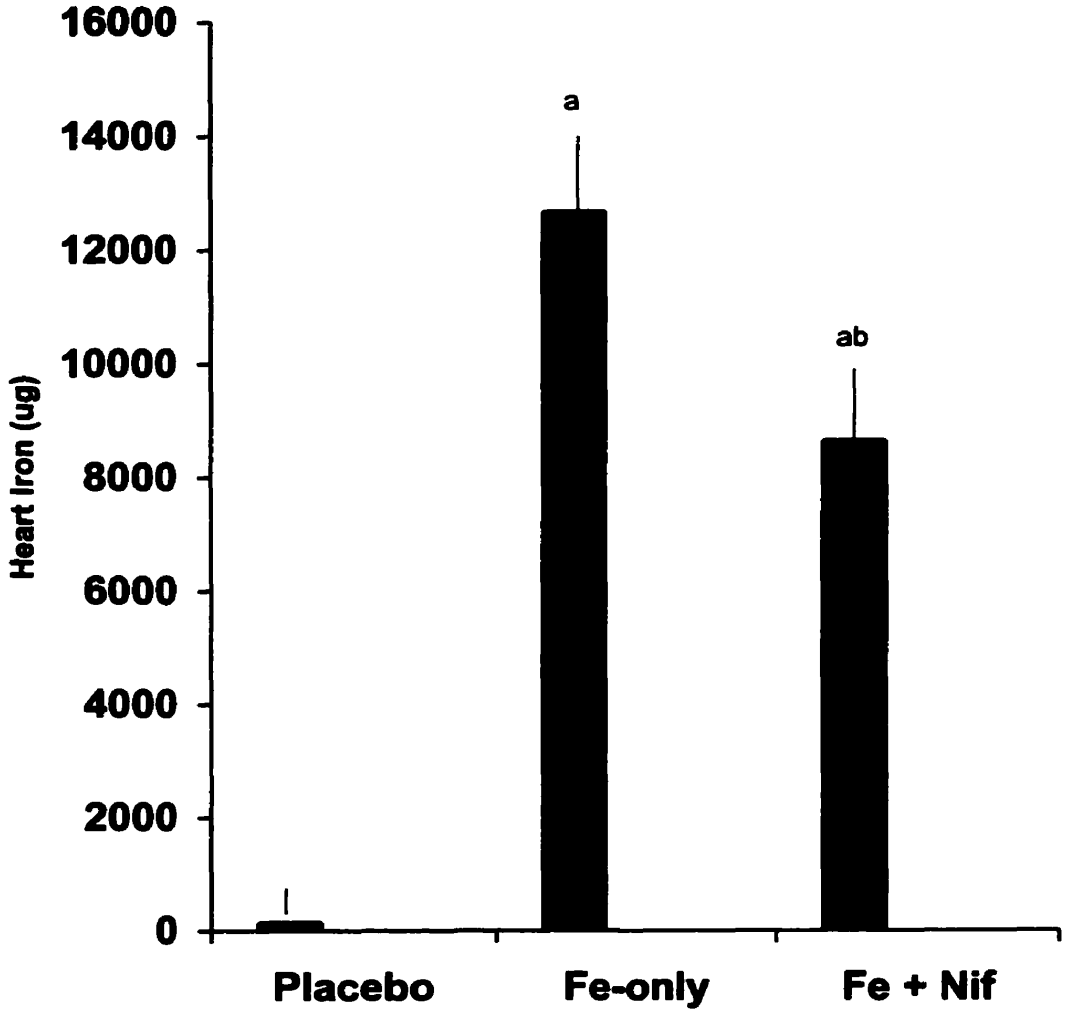

Figure 13: Heart tissue concentrations of iron (μg) as a function of treatment group. Note the decreased concentrations of iron present in the group treated with Nifedipine. All values are mean ± SD, a = $p < 0.05$ in comparison to vehicle controls, and b = $p < 0.05$ in comparison to Fe-only.

Heart tissue concentrations of the cytotoxic aldehyde hexanal are shown in Figure 14. In comparison to vehicle controls (4190 pica moles {pmoles} / 100 mg wet wt. tissue), a 216-fold increase (905092 pmols, $p < 0.05$) in hexanal concentrations is observed in iron-only treated mice, whereas a 8-fold increase (33848 pmols, $p = 0.17$) is noted in combined iron and nifedipine treated mice. In addition, when compared to iron-only treated mice, iron treated mice receiving supplementation with nifedipine had a 27-fold decrease ($p < 0.05$) in heart tissue concentrations of the cytotoxic aldehyde hexanal.

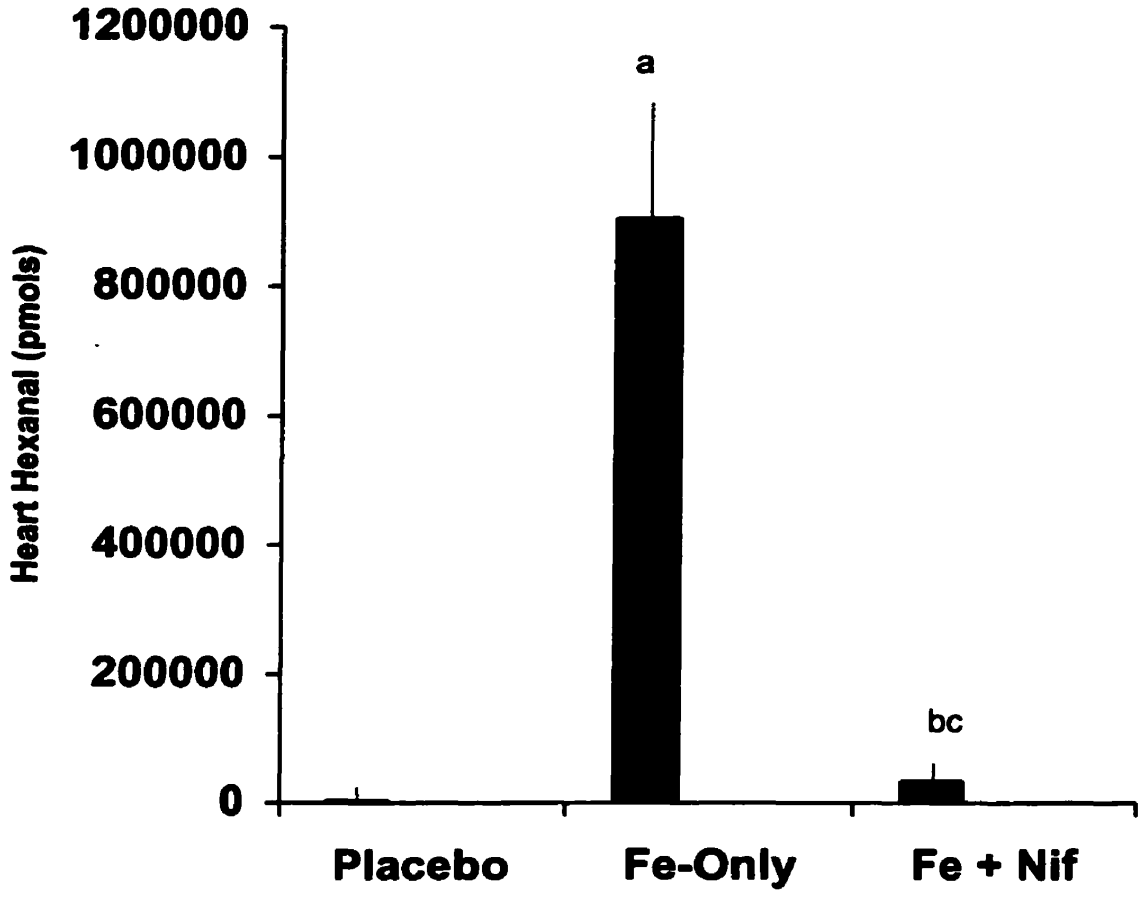

Figure 14: Heart tissue concentrations of hexanal (pmols/100 mg wet wt. tissue) as a function of treatment group. Note the decreased concentrations of hexanal in the Nifedipine treated group. All values are mean ± SD, a = $p < 0.05$ in comparison to vehicle controls, b = $p = 0.17$ in comparison to vehicle, and c = 0.05 in comparison to Fe-only

Figure 15 shows heart tissue concentrations of HNE by assigned treatment. In comparison to vehicle controls (1680 pmols), iron-only treated mice had a 6.7 fold increase (11183, $p < 0.05$) in heart tissue concentrations of the aldehyde HNE, whereas combined iron and nifedipine treated mice had a 14-fold increase (23616, $p < 0.05$). In comparison to iron-only treated mice, iron treated mice that received supplementation with nifedipine had a 2.1 fold increase ($p < 0.05$) in heart tissue concentrations of HNE.

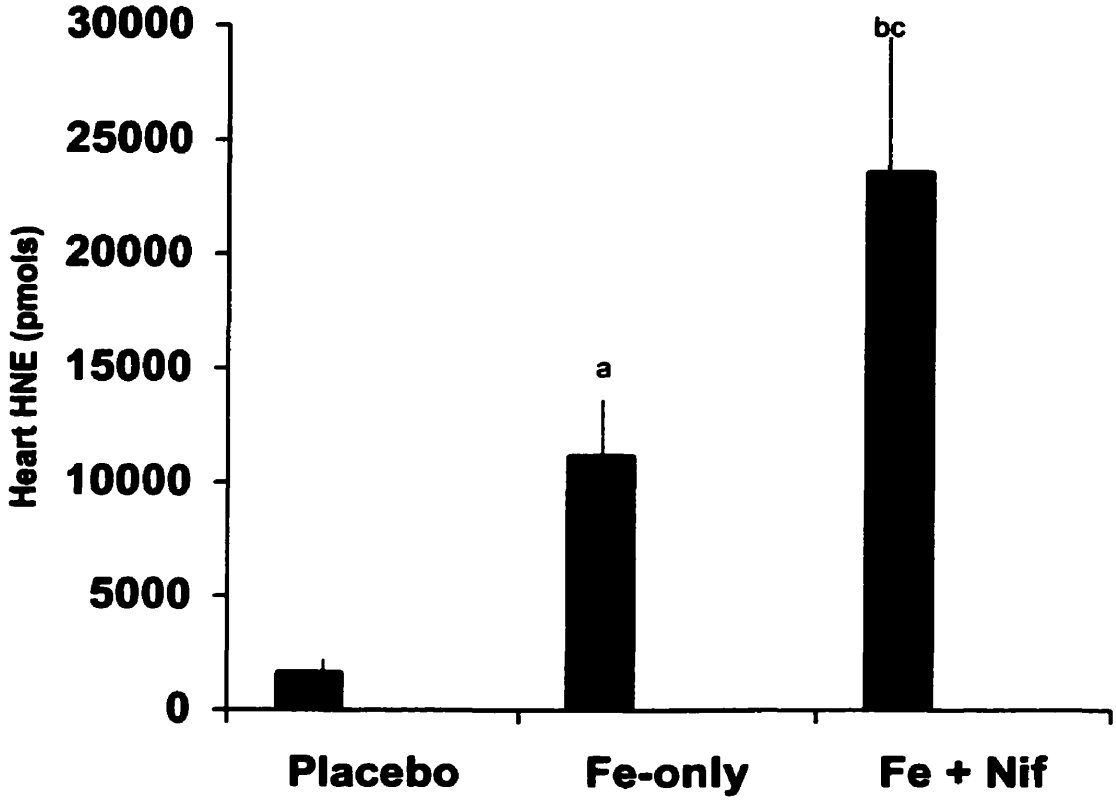

Figure 15: Heart tissue concentrations of HNE (pmols/100 mg wet wt. tissue) as a function of treatment group. Note that Nifedipine dose not decrease the production of HNE. All values are mean ± SD, a = $p < 0.05$ in comparison to vehicle controls, b = $p < 0.05$ in comparison to vehicle, and c = $p < 0.05$ in comparison to Fe-only

Figure 16 shows that heart tissue concentrations of MDA by assigned treatment. In comparison to vehicle controls (57957 pmols), iron-only treated mice had a 2.3 fold increase (135062 pmols, $p < 0.05$) in MDA concentrations; whereas combined iron and nifedipine treated mice had a 3.2 fold increase (186478 pmols, $p < 0.05$). Furthermore, combined iron and nifedipine treated mice had a 1.4 increase ($p = 0.18$) in heart MDA concentrations, when compared to iron-only treated mice.

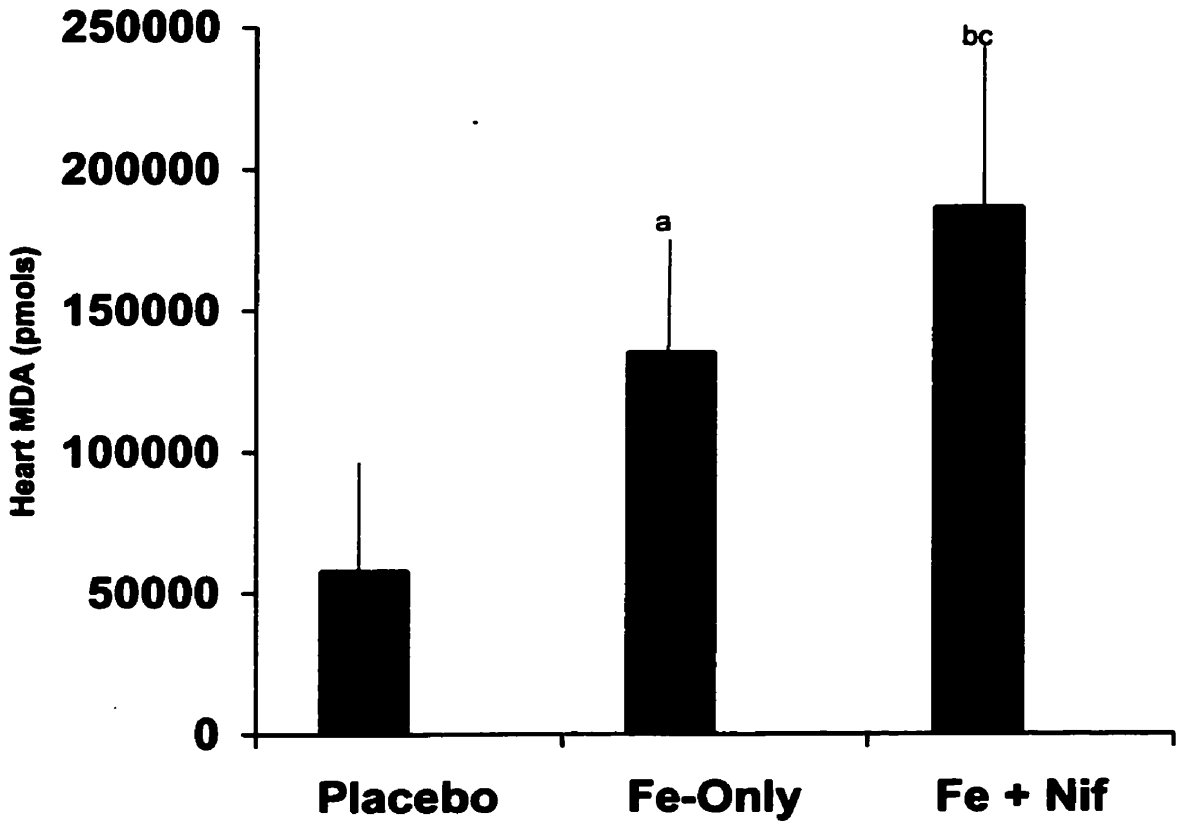

Figure 16: Heart tissue concentrations of MDA (pmols/100 mg wet wt. tissue) as a function of treatment group. Note that Nifedipine dose not decrease the production of MDA. All values are mean ± SD, a = $p < 0.05$ in comparison to vehicle controls, b = $p < 0.05$ in comparison to vehicle, and c = 0.18 in comparison to Fe-only

3.5 Discussion

HYPOTHESIS 3: Chronically iron-loaded mice that receive concurrent treatment with nifedipine will have decreased heart tissue concentrations of iron in comparison to iron-only treated mice.

HYPOTHESIS 4: Chronically iron-loaded mice that receive concurrent treatment with nifedipine will have decreased concentrations of cytotoxic aldehydes in comparison to iron-only treated mice.

In support of the third hypothesis the findings demonstrate that in fact, iron-uptake into the myocardium is partially inhibited by the administration of the dihydropyridine Ca^{2+} channel antagonist nifedipine. Under normal physiological conditions, iron uptake into the myocardium is mediated through transferrin bound mechanisms, whereas NTBI uptake in the heart is believed to play a minor role (DeSilva et al., 1994). Non-transferrin bound iron uptake has been shown to be enhanced by prior iron-loading of the cell, and uptake has been demonstrated in cardiac myocytes (Kaplan et al., 1991; Link et al., 1996). It has also been reported by Link and coworkers (1985) that NTBI uptake in rat cardiac cells can exceed iron uptake by transferrin-dependent processes by as much as 300-fold, with accompanying increases in aldehyde-derived peroxidation products (e.g. MDA). These results are consistent with previous findings that demonstrate that NTBI uptake under conditions of iron overload may be the primary mechanism of uptake during conditions of iron-overload (DeLuca et al., 1999; deValk et al., 2000; Gosriwatana et al., 1999, Tsushima et al., 1999). It has been suggested that NTBI uptake occurs because the capacity of serum transferrin to safely bind and detoxify "free iron" may be exceeded, thus the NTBI fraction of plasma may promote the

generation of toxic ROS such as hydroxyl radical formation resulting in damage to membrane lipids and proteins (deValk et al., 2000; Keher, 2000; Olivieri, 1999).

Aldehydes are end-products of lipid peroxidation employed as both markers and evidence of recent ROS activity in-vivo (Bartfay et al., 1999). Hence, an increase in the concentration of aldehydes in heart tissues implies increase ROS production in this experimental model.

The fourth hypothesis, which stated that iron-treated mice that received supplementation with nifedipine would have a lower production of ROSs in the heart, was not supported by the results in the present investigation. Although previous in-vitro and in-vivo studies have reported that nifedipine possesses significant antioxidant properties in various experimental systems (Engineer & Sridhar, 1989; Yao et al., 2000), no statistically significant differences were observed in nifedipine treated mice. These findings however were consistent with those of Sugawara and coworkers (1996) and Janero et al (1988) who reported that nifedipine had no statistically significant properties in comparison with other DHP calcium channel antagonists.

This was the first investigation to my knowledge to examine the antioxidant and iron-uptake blocking properties of nifedipine in an experimental model of chronic iron overload. Taken together, these preliminary findings are exciting because nifedipine has been shown to be partially effective in preventing iron-uptake in the chronically iron-loaded heart. Additional investigations are warranted to further elucidate the relation between heart concentrations of iron and the dose-dependent antioxidant and ROS-scavenging properties of nifedipine in the chronically iron-loaded heart. As well, additional investigations with higher dosages of nifedipine are required to clarify the

antioxidant properties of the agent in the chronically iron-loaded heart. Limitations of this study will be discussed concurrently with the amlodipine study in Chapter 6.

4.0 Chapter 4

Investigation III: Amlodipine intervention study

4.1 Aims and Rationale

The rational for the use of the L-type calcium channel blockers in a murine model of chronic iron-overload is the same as detailed in section 3.1. However, amlodipine was selected and studied because of its stronger lipophilic properties in comparison to other DHP's, thus raising the question as to whether it might exhibit different iron-blocking properties than nifedipine (Mason et al., 1999). The specific hypotheses addressed in this investigation were (i) chronically iron-loaded mice that receive concurrent treatment with the L-type calcium channel blocker amlodipine will have decreased heart tissue concentrations of iron in comparison to iron-only treated mice and (ii) chronically iron-loaded mice that receive concurrent treatment with the L-type calcium channel blocker amlodipine will have decreased concentrations of cytotoxic aldehydes in comparison to iron-only treated mice.

Method

4.2 Sample

The sample population and equipment were the same as detailed in Investigation I: Dose response study (see 2.2, p. 35).

4.3 Procedure

Iron loading was achieved by 20 mg (0.2 mL) i.p. injections of iron dextran on 5 of 7 days per week for a period of four weeks. Each group consisted of 5 mice per group for a total of three groups. The first group was the control group and received 0.2 mL of double distilled water (DDH_2O) i.p. per mouse per day. The second group was the iron

only group and received 20 mg of iron dextran i.p. per mouse per day. The third group was the iron plus amlodipine group. Specifically, 2.5 mg of Amlodipine was crushed and mixed into 200mL of drinking water along with 3.8 grams of sucrose for palatability. The solution was wrapped in tin foil to eliminated decomposition of the amlodipine due to light exposure and subsequently vortexed for 2-3 minutes to allow the solutes to dissolve. This mixture was changed twice a week (Monday and Wednesday) with no treatment occurring on weekends [see figure 17]. The rest of the study remained the same, including all endpoints, as detailed in Investigation I: Dose response study.

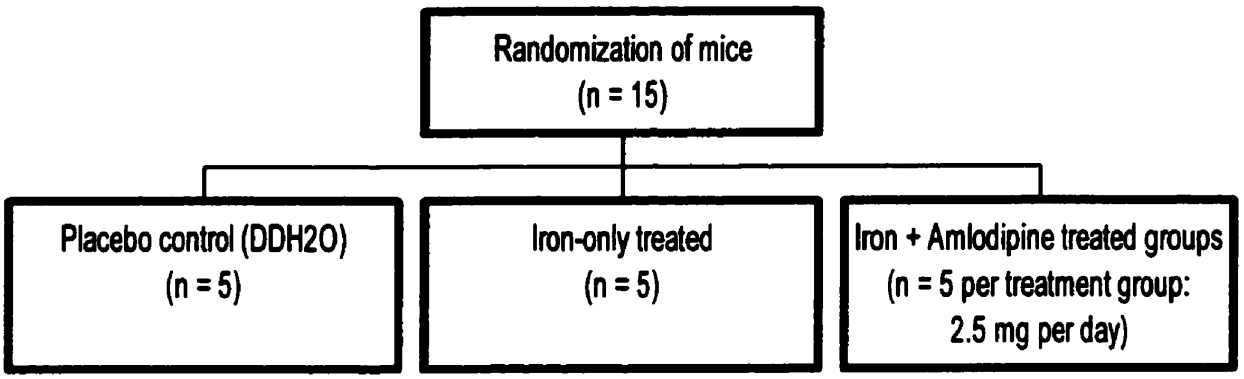

Figure 17: Amlodipine Intervention Study

4.4 Results

This investigation sought to determine the relationships between the total cumulative chronic dose of iron administered and the iron inhibiting as well as the antioxidant properties of amlodipine in the chronically iron-loaded heart. In comparison to DDH_2O treated mice, chronically iron-loaded mice had coarse fur with loss of colour and their major internal organs (liver, spleen, heart) appeared bronze in colour, when compared to the normal pink coloured organs in vehicle controls. Consistent with previous findings in this murine model, we observed that the mice became progressive lethargic and inactive with increasing total iron burden (Bartfay and Bartfay, 2000a, 2000b; Bartfay et al., 1998).

Figure 18 shows heart tissue concentrations of iron with assigned treatments. In comparison to vehicle controls (321 μg), iron-only treated mice (15272 μg) had a 47.6 fold increase ($p < 0.05$) in total iron concentrations; whereas iron and amlodipine treated mice (8474 μg) had a 26.4 fold increase ($p < 0.05$). Interestingly, in comparison to iron-only treated mice, iron-loaded mice that received supplementation with amlodipine had a 1.8 fold decrease ($p < 0.05$) in heart tissue concentrations of iron.

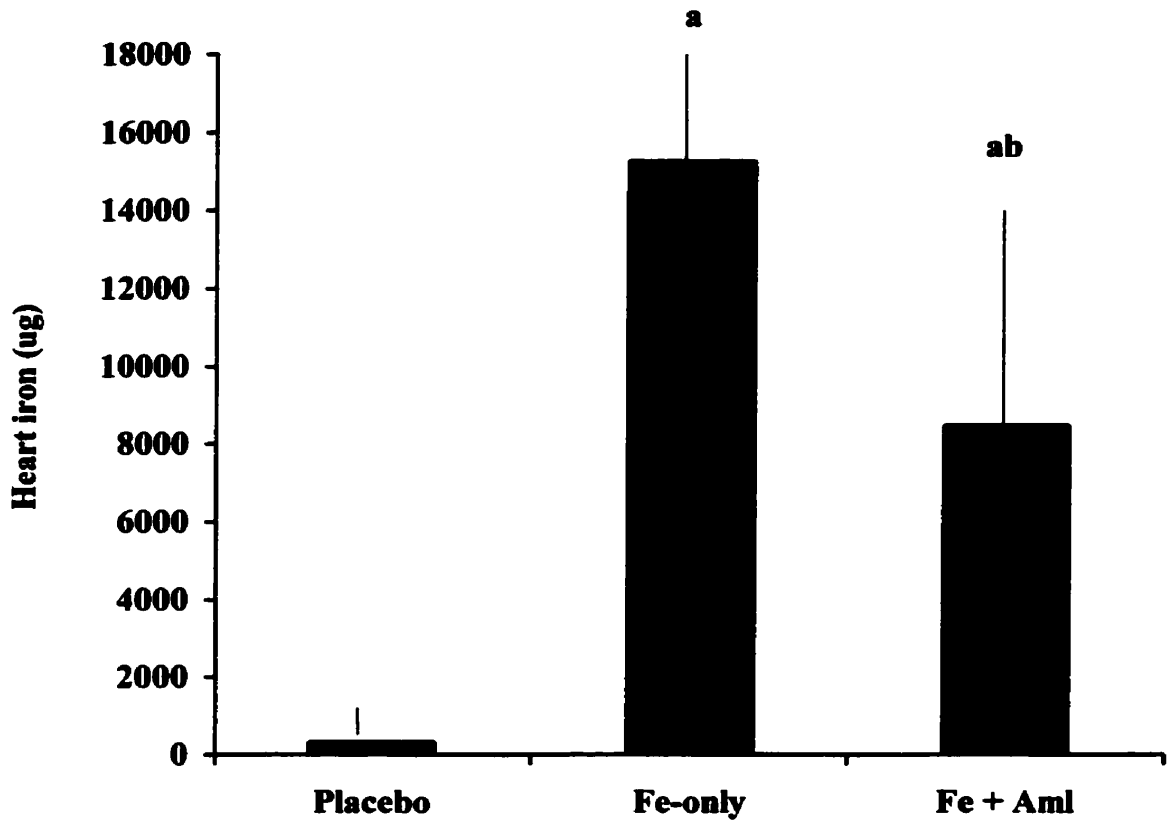

Figure 18: Heart tissue concentrations of iron (μg) as a function of treatment group. Note that Amlodipine decreases total heart iron when compared to Fe-only. All values are mean ± SD, a = $p < 0.05$ in comparison to vehicle controls, and b = $p < 0.05$ in comparison to Fe-only.

Heart tissue concentrations of the cytotoxic aldehyde hexanal are shown in Figure 19. In comparison to vehicle controls (17193 pica moles {pmols} / 100 mg wet wt. tissue), a 2.4 fold increase (41813 pmols, $p = 0.1$) in hexanal concentrations is observed in iron-only treated mice, whereas a 3.9 fold increase (68125 pmols, $p < 0.05$) is noted in combined iron and amlodipine treated mice. In addition, when compared to iron-only treated mice, iron treated mice receiving supplementation with amlodipine had a 1.6 fold ($p = 0.12$) increase in heart tissue concentrations of the cytotoxic aldehyde hexanal.

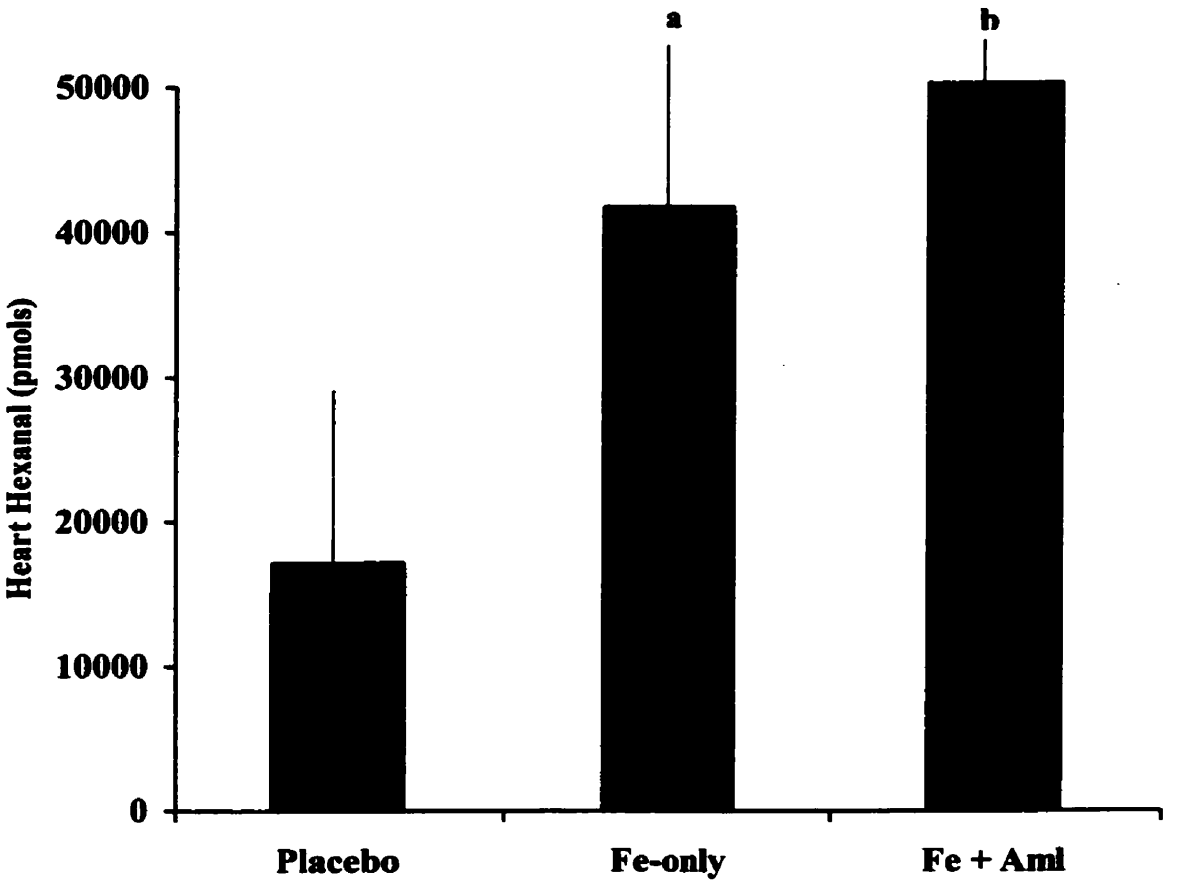

Figure 19: Heart tissue concentrations of hexanal (pmols/100 mg wet wt. tissue) as a function of treatment group. Note that concurrent administration of Amlodpine does not decrease hexanal concentrations. All values are mean ± SD, a = p = 0.1 in comparison to vehicle controls, b = p < 0.05 in comparison to vehicle

Figure 20 shows heart tissue concentrations of the HNE by assigned treatment. In comparison to vehicle controls (1091 pmols), iron-only treated mice had a 5.5 fold increase (6028 pmols, $p < 0.05$) in heart tissue concentrations of the aldehyde HNE, whereas combined iron and amlodipine treated mice had a 2.1 fold increase (2375 pmols, $p < 0.05$). In comparison to iron-only treated mice, iron treated mice that received supplementation with amlodipine had a 2.5 fold decrease ($p < 0.05$) in heart tissue concentrations of HNE.

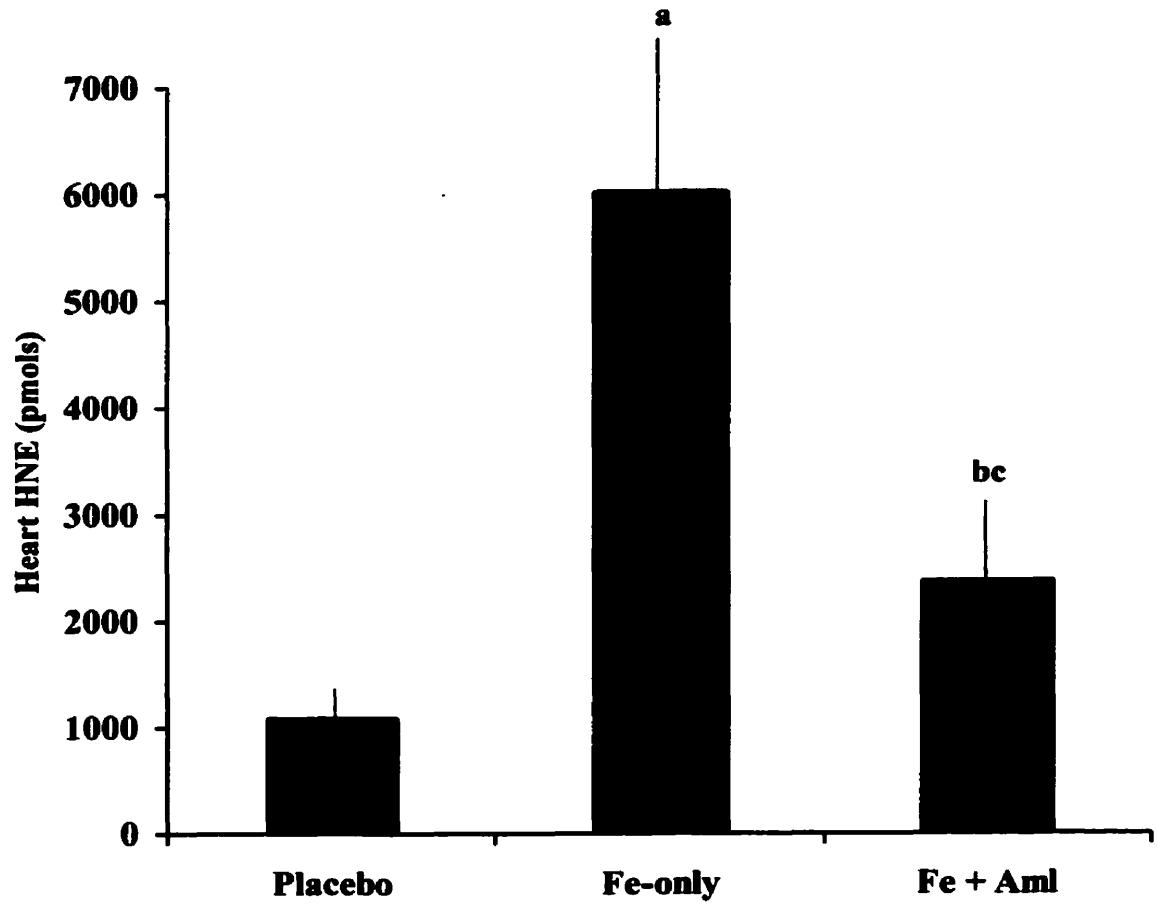

Figure 20: Heart tissue concentrations of HNE (pmols/100 mg wet wt. tissue) as a function of treatment group. Note that amlodipine decreases HNE concentrations in the heart compared to Fe-only. All values are mean ± SD, a = $p < 0.05$ in comparison to vehicle controls, b = $p < 0.05$ in comparison to vehicle, and c = $p < 0.05$ in comparison to Fe-only

Figure 21 shows the heart tissue concentrations of MDA by assigned treatment. In comparison to vehicle controls (40663 pmols), iron-only treated mice had a 3 fold increase (120127 pmols, $p < 0.05$) in MDA concentrations, whereas combined iron and amlodipine treated mice had a 1.6 fold increase (67636 pmols, $p < 0.05$). Furthermore, combined iron and amlodipine treated mice had a 1.7 fold decrease ($p = 0.159$) in heart MDA concentrations, when compared to iron-only treated mice.

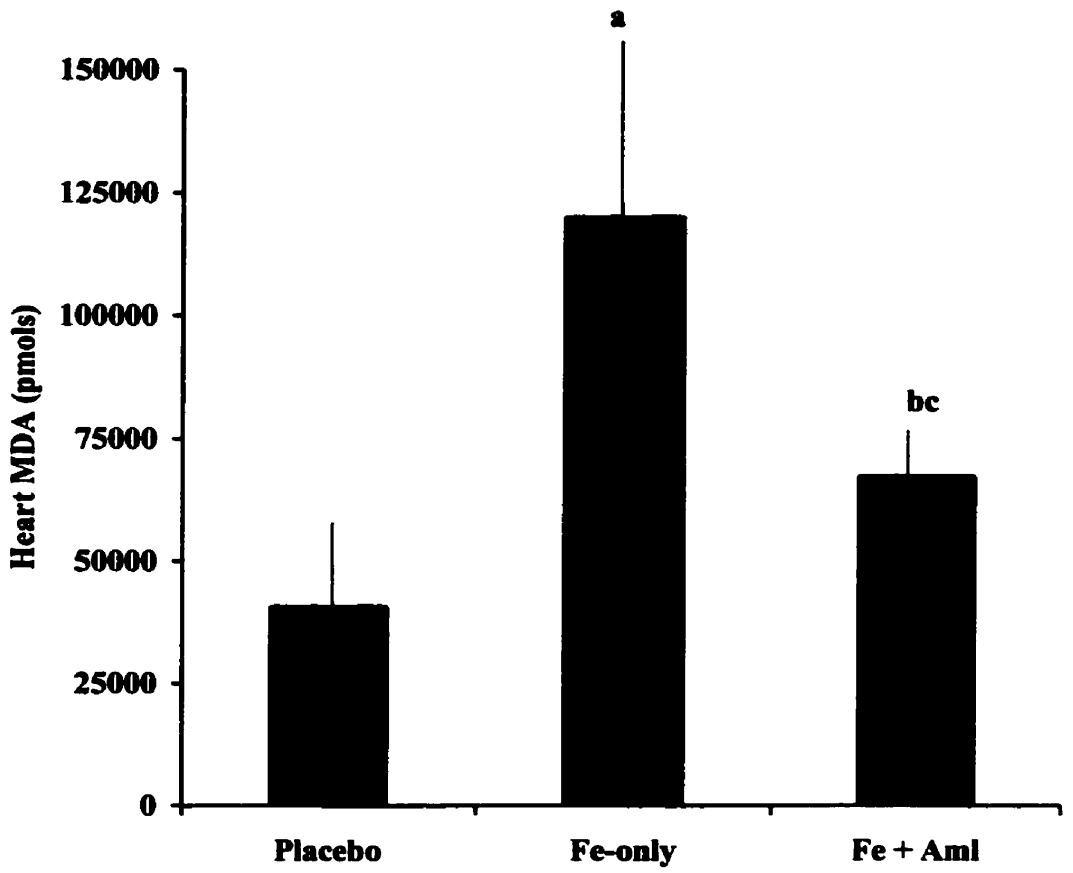

Figure 21: Heart tissue concentrations of MDA (pmols/100 mg wet wt. tissue) as a function of treatment group. Note that Amlodipine decreases MDA concentrations in the heart compared to Fe-only. All values are mean ± SD, a = $p < 0.05$ in comparison to vehicle controls, b = $p < 0.05$ in comparison to vehicle, and c = 0.159 in comparison to Fe-only

4.5 Discussion

HYPOTHESIS 5: Chronically iron-loaded mice that receive concurrent treatment with the L-type calcium channel blocker amlodipine will have decreased heart tissue concentrations of iron in comparison to iron-only treated mice.

HYPOTHESIS 6: Chronically iron-loaded mice that receive concurrent treatment with the L-type calcium channel blocker amlodipine will have decreased concentrations of cytotoxic aldehydes in comparison to iron-only treated mice.

In support of the fifth hypothesis, the findings showed that iron-uptake into the myocardium was partially inhibited by the administration of the dihydropyridine Ca^{2+} channel antagonist amlodipine. To my knowledge, this was the first study to examine the effects of the CCB amlodipine on iron-uptake in the heart of an in-vivo model of iron-overload cardiomyopathy (Crowe & Bartfay, 2001ab).

It has previously been reported that NTBI intake of the cell is enhanced by prior iron-loading (Kaplan et al., 1991; Randell et al., 1994), and its uptake has been demonstrated in cardiac myocytes (Link et al., 1996, 1985; Parkes et al., 1993). Under normal physiological conditions, iron uptake into the myocardium is mediated through transferrin bound mechanisms (DeSilva et al., 1994). Conversely, NTBI uptake in the heart is believed to play a minor role under normal physiological conditions, but may become the primary mechanism when serum iron concentrations are severely elevated as found in primary and secondary hemochromatosis (De Luca et al., 1999; de Valk et al., 2000; Gosriwata et al., 1999; Tsushima et al., 1999). Similarly, NTBI has been reported in the plasma of patients with primary and secondary hemochromatosis (De Luca et al.,

1999; de Valk et al., 2000; Gosriwata et al., 1999), and these patients also have increased plasma concentrations of ROS and decreases in protective antioxidant reserves (Bartfay and Bartfay, 2001; Bartfay et al., 1999c; Gutteridge et al., 1985; Livrea et al., 1996; Young et al., 1994). The toxicity of NTBI is much higher than of protein-bound-iron as evidenced by its ability to promote Fenton-type reactions resulting in peroxidative damage to membrane lipids and proteins (Demougeot et al., 2000; Kehrer 2000). In the heart, this results in impaired function of the mitochondrial respiratory chain and abnormal energy metabolism manifested clinically as hemosiderotic cardiomyopathy (Hershko et al., 1998; Liu and Olivieri 1994).

In support of the sixth hypothesis, iron-treated mice that received supplementation with amlodipine had significantly lower production of ROS in the heart, as quantified by the cytotoxic aldehydes hexanal, HNE and MDA. To my knowledge, this is the first report to examine the effects of the CCB amlodipine on heart tissue concentrations of iron and the production of ROS in an in-vivo model of iron-overload cardiomyopathy.

There is evidence to demonstrate in-vitro that L-type calcium channels contribute significantly to iron uptake by the heart and has many properties associated with the unknown NTBI uptake pathway resulting in an excess production of ROS (Tsushima et al., 1999). Congruent with these findings, there is a growing body of evidence to show that CCB's possess antioxidant properties that are entirely independent of Ca^{2+} channel modulation (Digiesi et al., 2000; Farghali et al., 2000; Sevanian et al., 2000; Sugawara et al., 1996). It is believed that the electron-rich aromatic ring of amlodipine is highly characteristic of most of the chain breaking antioxidants, such as α-tocopherol acetate

(vitamin E) (Mason et al., 1999). Hence, the antioxidant activity of the dihydropyridines, specifically lipophillic agents like amlodipine may have clinical relevance as an adjunct to chelators in the treatment and prevention of iron-overload cardiomyopathies.

Taken together, these are important preliminary findings because they suggest that some CCB's may have significance in the clinical management of iron-overload disorders, and offer for the first time a pharmacological vehicle to prevent the entry of excess iron into the heart of patients with disorders of iron metabolism. These findings, however, need to be interpreted with caution and are currently limited to a murine model of iron-overload cardiomyopathy. Further research is warranted to clarify the cardioprotective properties of CCB's and the exact mechanism(s) by which they limit iron-uptake in the myocardium and their specific antioxidant properties.

5.0 Chapter 5

General Discussion and Conclusion

Nursing is a dynamic profession that is developing a broader knowledge base through traditional forms of nursing research such as qualitative methodologies, but more recently through quantitative methodologies as found in basic research. Not only should the psychosocial aspects of life be a concern to the profession, but so should the understanding the biological mechanisms that result in pathology. Historically, nurses have been primarily interested in the stabilization of the patient and the prevention of clinical complications in an attempt to promote an optimal quality of life for a particular disease state. Therefore, the identification of a possible mechanism of injury and organ dysfunction resulting from chronic iron-overload is critical for the development of more effective treatments and interventions that help to stabilize and/or prevent clinical complications by nurses and other health care professionals. Furthermore, it is conceivable that that the identification of new pharmacological interventions in the treatment of iron-overload disorders could ultimately improve the quality and duration of life for affected individuals and their families.

In moderate quantities and attached to proteins, iron is an essential element in all cell metabolism and growth. However, in excess quantities and unleashed from proteins it is highly cytotoxic. Despite recent advances in the pharmacological management of chronic iron overload disorders such as β-thalassemia and hemochromatosis, iron-overload cardiomyopathy remains the leading cause of cardiovascular mortality worldwide in the second and third decades of life (Kontoghiorghes, 1995; Liu & Olivieri, 1994).

Although no single mechanism is likely to account for the complex pathophysiology of iron-induced heart failure, the toxicity of excess iron in the biological system is believed to be attributed to its ability to catalyze the generation of free radical species via Fenton-type reactions (McCord, 1996). According to the free radical hypothesis, an excess of free radicals and the accompanying decrease in antioxidant reserves play a key role in the pathogenesis of various clinical disorders such as heart disease, cancer, and diabetes (Halliwell, 1997; Gutteridge, 1993).

Consequently, it was one of the objectives of this thesis to determine if chronic iron-overload resulted in free radical mediated-injury in the heart, as quantified by the aldehyde-derived peroxidation products MDA, HNE, and hexanal. The second objective of this thesis was to determine the effectiveness of two separate L-type calcium channel blockers on preventing iron-uptake in the chronically iron-overloaded heart and their effectiveness of limiting the production of oxygen free radicals. Specific hypotheses were formulated to address the objectives of each study and these findings were evaluated above according to the results of the findings and the available empirical literature.

Limitations of the studies using nifedipine and amlodipine include only analyzing the iron and aldehyde concentrations in the heart and not analyzing liver or spleen concentrations, which are also susceptible to iron-overload. There are also limitations by the method in which oxygen free radical injury is measured. Aldehydes are a relatively stable and biologically active breakdown product lipid hydroperoxides (Esterbauer, 1993). Therefore, increased concentrations of aldehydes are regarded as both markers and evidence for recent free radical injury to lipid membranes in-vivo (Halliwell, 1997;

Cheeseman, 1993). Although the concentration of aldehydes is presumed to reflect free radical-mediated injury to lipid membranes it is conceivable that these measures may also reflect injury to proteins. Aldehydes such as HNE and MDA have been shown to bind with proteins and inhibit their function (Requena et al., 1996; Esterbauer et al., 1991). It has been reported that the detection of HNE and other unsaturated aldehydes from plasma and tissue varied between 60-80% as quantified by GC-MS (Luo et al., 1995). Despite these limitations, GC-MS is currently the method of choice for measuring aldehyde-derived peroxidation products in biological samples since it allows for a more precise measurement of various aldehydes simultaneously (Luo et al., 1995; Halliwell & Chirico, 1993).

Possible directions for further research include changing the route of administration of the calcium channel blocker from per os route of ingestion to intra-peritoneal injection to be able to clearly determine the precise amount of the CCB administered per mouse. A second possible direction for further research in a murine model of chronic iron overload is to combine treatment of CCB's with chelation therapy as chelation therapy is one known mechanism of excreting iron from the biological system (Bartfay et al., 1999). It would also be prudent to examine the ability of CCB's to preserve cardiac function employing a working perfused (Langendorff) model or by non-invasive echocardiography.

As evidenced by this study, not all CCBs exhibit the same hemodynamic and electrophysiologic properties. In particular there are marked differences between nifedipine and amlodipine (Gaviraghi et al., 1995). The ability of nifedipine to reduce afterload and induce coronary vasodilation, as well as to increase collateral blood supply,

has supported its use for angina pertoris. However, its short duration of action also provokes reflex tachycardia, which ultimately limits its beneficial effect and may precipitate pain. Newer dihydropyridine agents such as amlodipine and lacidipine are characterized by slow onset and long duration of vasodilatory activity and are able to reduce coronary resistance with little or no effect on heart rate. Mak and coworkers (1995) reported that calcium channel blockers, especially ones that are more lipophilic could provide cytoprotective effects that are important after oxidative injury and concluded that the antiatherogenic beneficial effects of calcium channel blockers may be related to their antiperoxidative activity.

Similar results regarding inhibition of iron uptake into the myocardium were reported when nifedipine was studied in-vitro by Savigni & Morgan (1996). They determined that nifedipine actually accentuated the uptake of non-transferrin-bound iron, but not transferrin bound iron even at low concentrations. However, they reported that nifedipine may act as an ionophore, with the capability of transferring Fe (II) across the membranes. If this activity is found to be specific for iron, it may provide a valuable adjunct in chelation therapy for iron overload. Since the photodegredation products of nifedipine lack Ca^{2+} channel blocking properties (Gurney et al., 1985; Sanguinetti & Kass, 1984), there should be no risk of side-effects due to actions of these channels. They concluded that nifedipine in conjunction with desferrioxamine was able to mediate iron release from erythroid cells at a far greater rate than was observed with desferrioxamine alone. Thus, nifedipine could provide a means of accentuating the excretion of excess body iron by desferrioxamine which is the only iron chelators that has achieved widespread clinical acceptance for this purpose.

Figure 22 below is a revised unified conceptual model of chronic iron-overload, which is based on findings from this thesis and the literature reviewed above. In comparison to Figure 6 (chapter 1, page 32), this thesis has significantly advanced our understanding of the effects of chronic iron-overload induced heart failure and the potential cardioprotective properties of calcium channel blockers. The conceptual model proposes that non-iron-loaded healthy individuals have "normal" metabolic free radical production. This basal metabolic concentration of free radicals reflects the normal balance between free radical generation and removal by antioxidant reserves (vitamin E, selenium). Ultimately, these healthy individuals have normal cardiac function and life expectancies for their respected age groups.

Conversely, a chronically iron-loaded heart which can result from β-thalassemia major or hemochromatosis have increased concentrations of cytotoxic aldehydes (Bartfay et al., 1999a). This reflects an imbalance between free radical generation and removal by the existing antioxidant reserves. It is proposed that this imbalance can lead to an increased risk of morbidity and mortality. Hence, by introducing a DHP into the equation, the administration of CCB's may block iron-uptake into the myocardium and may in fact alter the cascade of events, including decreasing the risk of morbidity and mortality in patients with genetic disorders of iron-metabolism. Interestingly, amlodipine may also possess antioxidant properties that may be even more beneficial to monitoring the integrity of the biological system than anticipated.

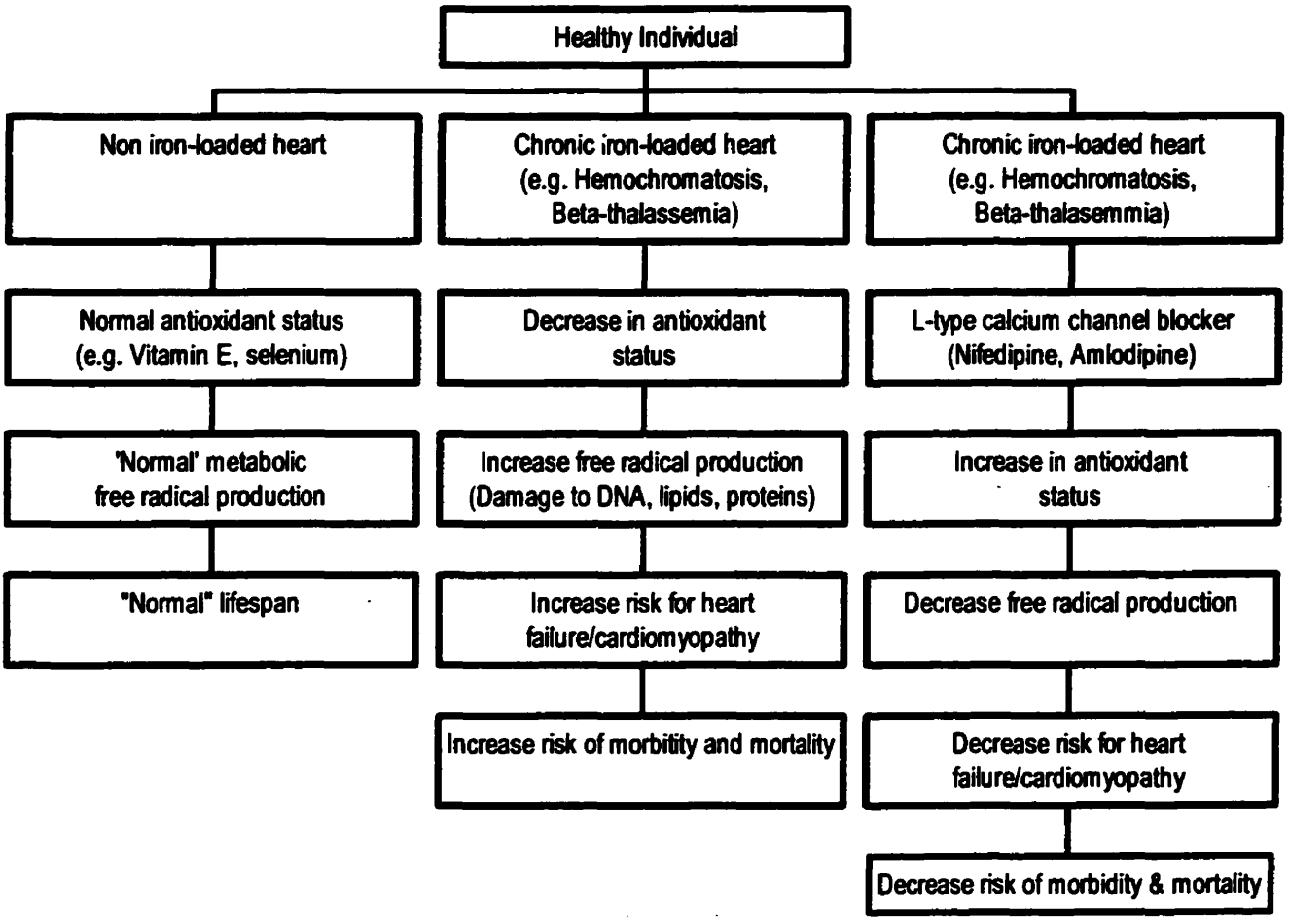

Figure 22: Proposed Conceptual Model of Chronic Iron-Overload. A revised conceptual model which proposes that L-type calcium channel blockers may be beneficial in interrupting the cascade of events by blocking iron-uptake in the heart due to its antioxidant properties, thus ultimately decreasing the risk of associated morbidity and mortality.

In summary, the identification that calcium channel blockers partially inhibits iron uptake into the myocardium and that amlodipine possesses antioxidant properties offers renewed promise for the clinical management and survival of patients with iron-overload disorders. Through our enhanced understanding of the pathogenesis of iron-induced heart failure, it is conceivable that new treatment modalities will emerge that could ultimately result in a better quality of life for affected individuals and their families.

6.0 References

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APPENDIX A

ANOVA Summary Table and Descriptive Statistics—Investigation I: Dose response study

Total Iron

	Mean	Std. Deviation	N
0 mg Fe	357.6000	49.17113	5
100 mg Fe	1507.8000	264.14333	5
200 mg Fe	4184.8000	400.31825	5
400 mg Fe	6826.8000	493.36569	5

Tests of Between-Subjects Effects

Measure: MEASURE_1

Transformed Variable: Average

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	207271411	1	207271411.3	2638.211	.000
Error	314260.500	4	78565.125		

Hexanal

	Mean	Std. Deviation	N
0 mg Fe	134.2000	10.42593	5
100 mg Fe	170.0000	8.94427	5
200 mg Fe	183.2000	20.95710	5
400 mg Fe	230.2000	37.32559	5

Tests of Between-Subjects Effects

Measure: MEASURE_1

Transformed Variable: Average

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	643687.200	1	643687.200	1771.657	.000
Error	1453.300	4	363.325		

HNE

	Mean	Std. Deviation	N
0 mg Fe	56.2000	9.52365	5
100 mg Fe	90.0000	6.81909	5
200 mg Fe	102.4000	7.05691	5
400 mg Fe	132.8000	8.58487	5

Tests of Between-Subjects Effects

Measure: MEASURE_1

Transformed Variable: Average

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	181832.450	1	181832.450	8010.240	.000
Error	90.800	4	22.700		

MDA

	Mean	Std. Deviation	N
0 mg Fe	1251.2000	55.96606	5
100 mg Fe	1857.8000	55.88112	5
200 mg Fe	2543.0000	240.32374	5
400 mg Fe	3915.2000	371.66679	5

Tests of Between-Subjects Effects

Measure: MEASURE_1

Transformed Variable: Average

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	114414145	1	114414144.8	4658.428	.000
Error	98242.700	4	24560.675		

ANOVA Summary Table and Descriptive Statistics—Investigation II: Nifedipine Intervention study

Total Iron

	Mean	Std. Deviation	N
Control	149.5000	23.01449	4
Fe-only	12678.00	2983.20845	4
Fe + 0.05g Nif	8642.2500	1257.94313	4

Tests of Between-Subjects Effects

Measure: MEASURE_1

Transformed Variable: Average

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	614600220	1	614600220.1	345.333	.000
Error	5339197.583	3	1779732.528		

Hexanal

	Mean	Std. Deviation	N
Control	3509.9267	1727.92208	3
Fe-only	905092.0	308446.84426	3
Fe + 0.05g Nif	42995.37	67621.17682	3

Tests of Between-Subjects Effects

Measure: MEASURE_1

Transformed Variable: Average

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	9.055E+11	1	9.055E+11	33.591	.029
Error	5.391E+10	2	2.696E+10		

MDA

	Mean	Std. Deviation	N
Control	72679.36	1817.85420	3
Fe-only	138578.9	23483.75687	3
Fe + 0.05g Nif	186477.9	104317.95550	3

Tests of Between-Subjects Effects

Measure: MEASURE_1

Transformed Variable: Average

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	1.582E+11	1	1.582E+11	36.085	.027
Error	8767868407	2	4383934203		

HNE

	Mean	Std. Deviation	N
Control	1744.0975	281.67865	3
Fe-only	11696.28	5523.49035	3
Fe + 0.05g Nif	23616.26	8493.22308	3

Tests of Between-Subjects Effects

Measure: MEASURE_1

Transformed Variable: Average

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	1830925597	1	1830925597	42.249	.007
Error	130009812	3	43336604.15		

ANOVA Summary Table and Descriptive Statistics—Investigation II: Amlodipine Intervention study

Total Iron

	Mean	Std. Deviation	N
Control	321.2500	103.94991	4
Fe-only	15272.00	4779.42172	4
Fe + 0.025g Aml	8474.2500	2935.66022	4

Tests of Between-Subjects Effects

Measure: MEASURE_1
Transformed Variable: Average

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	772326075	1	772326075.0	63.791	.004
Error	36321520.3	3	12107173.44		

Hexanal

	Mean	Std. Deviation	N
Control	17193.16	13623.66460	3
Fe-only	41813.42	25358.54732	3
Fe + 0.025g Aml	68124.74	23587.12357	3

Tests of Between-Subjects Effects

Measure: MEASURE_1
Transformed Variable: Average

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	1.616E+10	1	1.616E+10	24.289	.039
Error	1330845817	2	665422908.4		

MDA

	Mean	Std. Deviation	N
Control	41453.45	11495.99333	3
Fe-only	120127.1	92982.95261	3
Fe + 0.025g Aml	73139.98	28362.04026	3

Tests of Between-Subjects Effects

Measure: MEASURE_1

Transformed Variable: Average

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	5.509E+10	1	5.509E+10	26.557	.036
Error	4149045593	2	2074522796		

HNE

	Mean	Std. Deviation	N
Control	1091.2125	489.38101	3
Fe-only	6027.9575	3359.39713	3
Fe + 0.025g Aml	2375.0225	975.41627	3

Tests of Between-Subjects Effects

Measure: MEASURE_1

Transformed Variable: Average

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	120186255	1	120186255.0	31.868	.011
Error	11314175.6	3	3771391.881		