THE CRISIS OF IRON IN TRANSFUSION MEDICINE: IMPROVED IRON CHELATION THERAPY AND ITS IMPLICATIONS FOR CLINICAL PRACTICE IN THE MALDIVES

by

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Abstract

The Maldives has one of the highest incidences of β thalassemia in the world. Treatment of β thalassemia is characterized by two distinct phases: treatment of nature’s disease (anemia) and the secondary treatment of transfusional iron overload, a side effect of our cure. Interestingly, excess iron may also exert a negative effect on immune competence thus explaining the recurrent bacterial infections in these patients. Current iron chelation therapy using Desferal® (DFO) is challenging due to its short vascular half-life, frequency of injections, toxicity and expense. To address this problem, we have tested novel, low toxicity, high molecular weight (HMW) iron chelators. The utility of these chelators was tested in vitro using iron loaded (0-500 µM ferric (Fe³⁺) ammonium citrate, FAC; 0-48 hours) HepG2 and dendritic cells (DC). Iron chelation studies utilized either single or combinational treatment with deferiprone (L1) or DFO (both low molecular weight chelators) and S-DFO (a HMW derivative of DFO) for 0-48 hrs. The efficacy of treatment was assessed by cellular ferritin, Perl's iron stain, transmission electron microscopy (TEM), antigen presentation assays and cell viability assays. Iron treatment alone resulted in a significant increase in intracellular ferritin, histochemical iron staining and also resulted in a ~65.2% reduction in PBMC proliferation in response to the tetanus toxoid following 14 days of incubation. Treatment with either L1 or S-DFO alone demonstrated modestly decreased ferritin levels and iron staining. Importantly, combination therapy (L1+S-DFO) resulted in an additive effect resulting in a 79% decrease in FAC-driven ferritin levels after 48 hours and TEM studies of FAC treated, but not control, cells. FAC treated cells also demonstrated organellular and structural changes with electron dense iron deposits. As hypothesized, iron chelators (e.g., 200 µM DFO or L1) restored the PBMC proliferation in a concentration
dependent manner and reversed the ultra structural changes in organelles. The development of HMW chelators may provide better therapeutic value (reduced toxicity and less frequent administration) in developing nations. Consequent to this, iron mediated pathology in patients would be diminished, resulting in less cost to already strained public health budgets such as in the Maldives.
Preface

Following informed consent, human blood samples were collected from normal volunteer donors for this research as approved by the UBC Office of Research Services certificate number H0270215-A005.
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<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
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<td>BMT</td>
<td>Bone Marrow Transplantation</td>
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<td>National Thalassemia Centre</td>
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<td>NGO</td>
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<td>Peripheral Blood Mononuclear Cell</td>
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<td>Thiobarbituric Acid-Reactive Reactive Substances</td>
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<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
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<td>TEM</td>
<td>Transmission Electron Microscopy</td>
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<td>World Health Organization</td>
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Finally but not least, I am always grateful to have wonderful parents, family and friends to support me for everything I go through in life.
Dedication

To my parents, Hassan Ibrahim and Khadheeba Hussain
Chapter 1: Background

1.1 Overview

The island nation of Maldives is a world ‘hot spot’ for thalassemia. The prevalence of β-thalassemia in the population of this country is 18.1%; 1 in 5 people are carriers of the disease and 1 in 120 live births are estimated to be thalassemia majors. Currently, there are more than 700 thalassemia major patients who require regular blood transfusions, and as a result, 60% of the blood utilization of the Maldives treats these patients.

The pathophysiology of β-thalassemia divides into two distinct disease processes, one caused by nature (mutations; primary disease) and one caused by treatment (blood transfusions; secondary disease). The release of heme and iron from the unstable hemoglobin within the erythrocyte results in the premature destruction of the cell and a mild to severe anemia. While transfusions correct the anemia, secondary iron overload occurs, counterproductive to chronic transfusions. Both of these mechanisms are characterized by disease processes that arise in part due to iron-driven redox reactions. Thus, both the primary and secondary pathology of thalassemia arise from “misplaced” iron.

Removal of oxidatively damaged thalassemic RBC in vivo occurs primarily via erythrophagocytosis by the mononuclear phagocytic system (MPS). This clearance mechanism may result in negative immunoregulatory effects on these patients, such as an increased risk of bacterial infections. We sought to investigate the many pathologies involved in thalassemia due to “misplaced iron” that can be minimized by higher quality iron chelating agents. In this thesis, I will investigate a common denominator to the practical issues facing transfusion medicine, clinical practice, and the implications of improved iron chelation therapy in the Maldives. I will argue that strengthened iron chelation therapies may be significant in improving transfusion medicine practices in developing nations such as the Maldives.
1.2 Transfusion Medicine in the Third World – The Maldives Experience

In underdeveloped countries, especially in Asia and Africa, the supply of blood does not meet the demand due to the risks of infectious diseases, prevalence of genetically inherited blood disorders and primitive blood banking facilities. Blood utilization priority in some Asian countries goes to transfusion-dependent patients, such as β-thalassemia majors, those with sickle cell disease and HbE β-thalassemia. In South East Asia, for example, current blood collections comprise 7 million units, while the rest of the world collects 68 million units; this blood collection is less than 50% of the 15 million units required to meet the global shortage of blood. (Figure 1.1) (1). Africa is similar in that needs vastly outweigh the available resources. In the Maldives, ~11,000 units of blood are collected annually and 60% of the collection is allocated to thalassemia major patients.

Blood transfusions save lives, and yet millions of patients requiring transfusions do not have timely access to safe blood. Every nation should provide safe blood that is free from transfusion transmissible diseases, develop preventive measures for future risks, and provide treatment for the adverse effects of blood transfusions, such as secondary iron overload. However transfusion medicine in the third world bears little resemblance to current practices in North America and Western Europe.

Figure 1.1. Blood collection and utilization in South East Asia and the rest of the world. Shown is A population and B blood collection, current blood collections are <50% of those needed to meet the demands of South East Asia. Data derived from R.Sharma, BMJ 2000 (1).
1.2.1 Saga of Thalassemia: Maldives Outlook and the At-Risk Population

The Maldives (Figure 1.2) is one of the many South Asian countries experiencing an enormous problem with blood supply and demand, and along with Cyprus, is one of the world’s top thalassemia “hot spots.” (2). The origins of the Maldivians are uncertain. Nevertheless, it is believed that their history goes as far back as BC 1000. Early settlers were travelers on the Silk Route from civilizations in the Indus Valley. Although it has been hypothesized that the genetically inherited heterogeneous disease “thalassaemia” may have been brought to the Maldives through traders’ routes, there is no significant scientific evidence to prove this, nor is there any scientific proof that defective genes are a direct result of mutations within the Maldivian population. While very common historically, the earliest case of medically diagnosed thalassemia in the Maldives only goes back to the early 1970s. In the decade spanning 1970 to 1980, few cases were registered in the government hospital because there was no proper regimen for transfusion and management of the disease during this period. Ree published the earliest study on thalassemia in the Maldives in 1977 (3). Ree investigated anemia in the population of Addu, the southernmost atoll of the Maldives, reporting that β-thalassemia minor existed in 10.1% of the 352 individuals examined and that an additional 10% of the subjects suffered from iron deficiency anemia; although Ree found no malarial parasites in the blood films, 26 people were positive for malarial antibodies by ELISA (3). During 1941-1976, the British Royal Air Force base on Gan Island of the Addu atoll provided medical service to the people in that area.
Figure 1.2. World Distribution of α- and β-Thalassemia. It is estimated there are 270 million thalassemia carriers in the world, 190 million α and 80 million β- thalassemia carriers. Maldives has the highest percentage of thalassemia carriers in the world. Also indicated are the two major cities of the Maldives: Male and Addu. World map is from the font webdings, Maldives map is not proportional to the world map. Figure is original artwork (Mustafa © 2010) (4).

The infant mortality rate in the Maldives was 60 per 1000 in 1985, as a partial consequence of this stage-inherited disease that emerged as a public health issue. During 1987, thalassemia was such an important issue in the Maldives that the government contacted the World Health Organization (WHO) for advice. Acting on a request from WHO in 1988, Dr. Bernadette Modell, along with other British researchers and in collaboration with some local staff, conducted a study on the identification of hemoglobinopathies in the Maldives (5). The initial screenings were achieved through a very simple method: the one tube osmotic fragility test (OFT) with 0.36% buffered saline (6). After these preliminary investigations, they found that a fairly high percentage of samples were positive for OFT, but there were no facilities for proper laboratory diagnosis of hemoglobinopathies in the Maldives.
With these initial findings, WHO consultants highlighted the need for a national thalassemia-screening program. During 1980-1990, there was a dramatic increase in diagnosed thalassemia patients in the country because of awareness campaigns conducted by non-government organizations (NGOs) and the Maldivian government, who set up special clinics for these patients in the pediatric wards of hospitals. The government, recognizing the negative impact of thalassemia and its social stigma in the affected communities, brought in many experts from various countries in collaboration with WHO during the 1980s. In 1989, the molecular basis of β-thalassemia in the Maldives was studied by a Japanese research group at Kyushu University, in collaboration with the Thalassemia Research Center, Institute of Science and Technology for Research and Development at Mahidol University in Thailand and the Maldives Thalassemia Prevention Program. The study identified 4 common mutations of thalassemia in the Maldives (7). (Table 1.1) Surprisingly, these findings suggest that mutations in the Maldives do not resemble the common mutations frequently found in neighboring countries in India and South East Asia. The study did indicate, however, that some of these common mutations found in the Maldives were comparable to those found in Indonesia and Melanesia (7).

Table 1.1 β-thalassemia mutations in the Maldives. (7)

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Number of Alleles</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVS-I-5 (G C)</td>
<td>58</td>
<td>74.3</td>
</tr>
<tr>
<td>Codon 30 (AGG ACG)</td>
<td>12</td>
<td>15.4</td>
</tr>
<tr>
<td>IVS-I-1 (G A)</td>
<td>7</td>
<td>9.0</td>
</tr>
<tr>
<td>Codon 41/42 (-TT CT)</td>
<td>1</td>
<td>1.3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>78</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>

With the further assistance of WHO experts and prompted by the high incidence of thalassemia in the Maldives, the National Thalassemia Center (NTC) was inaugurated in 1994. The center’s mandate was to carry out the National Thalassemia Programme (NTP), with
components that included thalassemia screening, genetic counseling, and treatment for thalassemia major patients. The extensive hemoglobinopathy screening program was established with the aid of modern diagnostic laboratory methods, including hemoglobin electrophoresis, automated blood cell counts, and microscopy, as well as hemoglobin quantification by high performance liquid chromatography (HPLC) methods. By 2004, more than a quarter of the national population were screened for hemoglobinopathies, and the mean prevalence of β-thalassemia was established at 18.1% with significant inter-island variations; nine islands have a prevalence above 30% and twenty-eight islands have a prevalence of more than 25%. (Table 1.2) The Society for Health Education (SHE) in collaboration with the Government of Maldives carried out this research (8). Maldives is now considered to have the highest percentage of β-thalassemia carriers in the world. Indeed 1 in 5 people are carriers and 1 in 120 births result, estimated to be a thalassemia major patient (8).

A study conducted on umbilical cord blood in the Maldives shows 28% of 200 samples are α-thalassemia carriers. The enormous contribution of many people in both the government and NGOs across the country has dramatically raised thalassemia awareness, and today “thalassemia” is a household word in Maldivian communities.
Table 1.2 Prevalence of thalassemia in Asia where information is available.

<table>
<thead>
<tr>
<th>Country</th>
<th>Average carrier rate % for common thalassemia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha^0$-thalassemia</td>
</tr>
<tr>
<td>India</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Sri Lanka</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Maldives</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Myanmar</td>
<td>NA</td>
</tr>
<tr>
<td>Thailand</td>
<td>2.2-9</td>
</tr>
<tr>
<td>Cambodia</td>
<td>1</td>
</tr>
<tr>
<td>Laos</td>
<td>NA</td>
</tr>
<tr>
<td>Vietnam</td>
<td>NA</td>
</tr>
<tr>
<td>Southern China</td>
<td>15</td>
</tr>
<tr>
<td>Hong Kong</td>
<td>2.2</td>
</tr>
<tr>
<td>Taiwan</td>
<td>5</td>
</tr>
<tr>
<td>Singapore</td>
<td>2-3</td>
</tr>
<tr>
<td>Malaysia</td>
<td>4.5</td>
</tr>
<tr>
<td>Indonesia</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Philippines</td>
<td>5</td>
</tr>
</tbody>
</table>

Data modified from the first Meeting of the Asian Network for Thalassemia Control in Bangkok, Thailand, 3-4 July 2005. *$\alpha^+$ thalassemia in the data is a DNA study on 200 samples cord blood (8, 9).

1.2.2 Geographical Challenges

The Maldives is a country with a distinctive and challenging geography. A small island nation with 20 natural atolls located in the Indian Ocean, south-south west of India, the total landmass of the 1190 small coral islands of the archipelago is less than one percent of its national territory (Figure 1.3). The necklace of islands around the atolls varies, from single islands in some atolls to many islands in others. As the country's 2009 population census shows, a total of 396,334 people live in the country; of the 1190 islands only 200 are inhabited. The population of the 200 inhabited islands varies considerably, with an average population of 1000. However, 76 islands have less than 500 inhabitants (US State Department).
Figure 1.3. Maldives: a model of a small island nation with a distinctive geography. The total landmass of the 1192 small islands of the country is less than one percent of its geographical territory. The population of the 200 inhabited islands varies considerably. The country's 2009 population census shows a total of 396,334 people living in the country. Facts from Maldives Department of National Planning. Figure is original artwork (© Mustafa, 2010).

The equator passes through the Huvadhu Atoll of the Maldives. (Figure 1.3) The regions that are north of the equator are affected by a monsoon climate. Strong northeast winds blow from October until April; from May until October, south and west winds prevail. Crossing the Arabian Sea, the violent monsoons bring rain to the Indian subcontinent. In the southern hemisphere, the winds are generally milder, but summer storms near Mauritius can be severe. When the monsoon winds change, cyclones sometimes strike the shores of the Arabian Sea and the Bay of Bengal (10).

Transportation between islands and inter atolls in the Maldives is a considerable challenge. The weather and reef configuration patterns obstruct the accessibility of many islands, especially during the monsoon season. Travelling throughout the Maldives is expensive.
and it is sometimes difficult to reach remote islands. The principal forms of transportation in the Maldives are mechanized boats and limited aircraft, either seaplanes or small aircraft travelling to some regional airports. All these issues adversely affect transfusion medicine and patient access to the limited blood supply in the Maldives.

1.2.3 Financial Challenges

Transfusion medicine also faces financial hurdles in the Maldives, where the 2009 official GNI (gross national income) per capita is US$3,970 according to the World Bank, a figure that is relatively high compared to most South Asian countries. The disparity of wealth distribution, however, is not reflected in this official data. At the moment, transfusion for β-thalassemia major patients in the Maldives is provided by the government at no cost. By 2009, NTC spent US$1.12 million for thalassemia screening and treatment (11). It is estimated the average annual cost exceeds US$6,000 per patient and another US$13.26 for an individual hemoglobinopathy screening test. Thalassemic children require continuous care and treatment to stay alive. They require regular blood transfusions and iron chelation therapy with the drug Desferal®, which is injected five times a week, or with oral chelators, Ferriprox® and Exjade®.

At present the only permanent cure for thalassemia major is an allogeneic stem cell bone marrow transplant (BMT). However, the cost of this treatment ranks between US$20,000 and US$25,000 in some of the countries close to the Maldives, such as India and Thailand (12,13). Due to the low income of most people in the Maldives, this costly BMT treatment is not accessible for many families. Those who can afford the treatment undergo it abroad, as it is not available in the Maldives.

Despite these challenges the country is progressing both socially and economically in its ability to provide higher-quality health care. The country has managed to reduce infant mortality
from a rate of 60 per 1000 in 1985 to 11 per 1000 in 2009 (11). In addition, malaria is eradicated from the Maldives, and 95% of children are immunized. (WHO, Maldives Country Report 1995)

1.2.4 Current Transfusion Medicine Practice in the Maldives

Currently there are about 700 thalassemia major patients registered who receive regular red blood cell (RBC) transfusions, mostly at NTC located in the capital island of Male. (Figure 1.4) A daily average of 25-30 thalassemia patients receive blood transfusions at NTC. However, the available resources do not provide adequate infrastructure and facilities to supply safe blood for these patients, much less for other demands. At present, 60% of the blood collection is allocated to thalassemia major patients, while 40% remains for other patients’ needs. Since there is no organized and centralized blood banking system in the Maldives, other blood products are not readily available, especially plasma products (e.g., fresh frozen plasma, cryoprecipitate), and platelet concentrates, that are occasionally crucially needed to treat patients in tertiary hospitals. These patients are customarily referred to the neighboring countries of Sri Lanka or India. Blood donation in the Maldives remains voluntary and is mostly recruited by the patients themselves, from friends and family.

The standard thalassemia treatment provided by NTC is focused on maintaining an adequate hemoglobin level in patients. In addition, to treat secondary iron overload Desferal® is provided to the patients, and limited numbers of patients are on L1 treatment as well (14). However, the compliance of patients with transfusion and iron chelation therapy is not satisfactory: only 65% of patients get regular transfusions to maintain hemoglobin at 9-10 gm%, and only 50% of patients comply with iron chelation therapy guidelines. (Figure 1.5)
Figure 1.4. Incidence of β-thalassemia major in the Maldives. There are about 700 cases that have been registered at NTC. The number of new cases registered has not declined over the last five years, with about 28 new cases on average being registered over the last 7 years. In 2008, this number has increased, with 39 new cases diagnosed and registered. Data from National Thalassemia Centre, Maldives 2008.

Blood safety is an essential component of modern transfusion medicine. In the Maldives blood is screened for transfusion transmissible infections (TTI), including HIV1 and 2 antibodies, hepatitis B (HbsAg), HCV, and syphilis (VDRL). At present, rapid test kits carry out these tests for ELISA or EIA due to the small number of donors and lack of technical expertise in these islands. Although it is highly recommended and widely accepted that thalassemics be treated with pre-storage leukocyte-depleted packed RBC, in the Maldives leukocyte depleted blood is not readily available, and thus it is only an option for patients who can afford to buy their own bedside leukoreduction disposable filters. Acute non-hemolytic febrile transfusion mediated reactions are common among patients who get regular blood transfusions due to alloimmunization. Since 1999, the Supreme Council of Islamic Affairs in the Maldives legalized prenatal diagnosis (PND), medically terminated pregnancy (MTP), premarital counseling and screening for high risk couples as a legal requirement (8).
Compliance of iron chelation therapy and current clinical practice of treatment for β-thalassemia major in the Maldives. The data shows that 65% of patients get regular transfusions to maintain their hemoglobin level at 9-10 gm%, while 35% of patients have inadequate transfusions and a hemoglobin level below 8 gm%. Only 50% of patients complied with DFO treatment, 35% were graded as poor compliance, while 15% show very poor compliance or sometimes do not receive iron chelation therapy. Data personally collected from NTC, Maldives 2004.

1.2.5 What Can be Done in Light of These Challenges?

A common denominator to the practical issues facing the Maldives in the treatment of thalassemia is the role of iron in disease pathology. Iron mediated injury underlies both the initial anemia and the essential iron chelation therapy in transfused patients. Although iron chelation therapy is the most practical solution for a large number of thalassemia patients around the world; very few people have access to this treatment, especially in areas where thalassemia is highly prevalent. (Table 1.3) For the Maldives, the challenges are enormous when viewed in light of the available resources, geography, and high incidence of disease.

Improved high molecular weight (HMW) iron chelators exhibiting increased plasma retention time may have substantial therapeutic benefits. If this new improved classes of high molecular weight chelators are successful, their administration via daily oral chelators with a weekly or bi-monthly injection of HMW chelators could provide new possibilities for the
improved management of thalassemia major in developing countries. Furthermore, these improved iron chelators might have other applications in the areas of blood banking, such as improving the quality and storage shelf life of blood products, especially platelet concentrates, which are currently not available in the Maldives. Hence, improved chelation therapy may provide significant benefits to nations such as the Maldives.

Table 1.3 Reach of treatment for thalassemia in the world. (14)

<table>
<thead>
<tr>
<th>WHO Region</th>
<th>Estimated annual birth rate for β thalassemia</th>
<th>Transfusion</th>
<th>Adequate iron chelation</th>
<th>Inadequate or no iron chelation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>% of transfusion</td>
<td>% with chelation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>dependent</td>
<td>dependent patients</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>transfused</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Africa</td>
<td>1386</td>
<td>1278</td>
<td>2.7</td>
<td>1243</td>
</tr>
<tr>
<td>America</td>
<td>341</td>
<td>255</td>
<td>52.4</td>
<td>121</td>
</tr>
<tr>
<td>East Mediterranean</td>
<td>9914</td>
<td>9053</td>
<td>17.8</td>
<td>7443</td>
</tr>
<tr>
<td>Europe</td>
<td>1019</td>
<td>920</td>
<td>15.5</td>
<td>780</td>
</tr>
<tr>
<td>South East Asia</td>
<td>20420</td>
<td>9983</td>
<td>9.6</td>
<td>9021</td>
</tr>
<tr>
<td>Western Pacific</td>
<td>7538</td>
<td>4022</td>
<td>2.7</td>
<td>3914</td>
</tr>
<tr>
<td>World</td>
<td>40618</td>
<td>25511</td>
<td>11.7</td>
<td>22522</td>
</tr>
</tbody>
</table>

* Data is minimum estimation

1.3 Where is “Misplaced” Iron in Transfusion Medicine?

1.3.1 “Primary” Iron Overload-Misplaced Iron Within the RBC

Surprising to some the majority of iron in the human body is located within the red blood cells (RBC). The normal RBC is the most ferruginous somatic cell, containing approximately 20 mM iron (15). Each erythrocyte contains >1 billion atoms of iron in hemoglobin; at normal turnover rates, this corresponds to incorporation of $2 \times 10^{20}$ atoms of iron per day (16). Indeed, approximately 67% of the iron in the human body is in the form of hemoglobin and myoglobin.

Hemoglobin is a 64,000 Dalton (Da) tetramer protein composed of two α and two β like globin polypeptide chains, each non-covalently associated to an iron-containing heme prosthetic
group (17). During development fetal hemoglobin (HbF), $\alpha_2 \gamma_2$ is predominant during 10-12 weeks post conception. The $\gamma$ chain is replaced by $\beta$-globin subunits, which become principal with $\alpha$ chains throughout adult life. Normal adult hemoglobin consists of HbA that is $\alpha_2 \beta_2$ tetramers, with minor components HbA$_2$ comprised of $\alpha_2 \delta_2$ chains. A normal adult has HbA proportion of 97%, HbA$_2$ ~2.5% and trace amounts of HbF (18). (Table 1.4)

Table 1.4. Normal human hemoglobin. (19)

<table>
<thead>
<tr>
<th>Name</th>
<th>Designation</th>
<th>Molecular structure</th>
<th>Proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Adults (%)</td>
</tr>
<tr>
<td>Adult hemoglobin</td>
<td>A</td>
<td>$\alpha_2 \beta_2$</td>
<td>97.0</td>
</tr>
<tr>
<td>Hemoglobin A$_2$</td>
<td>A$_2$</td>
<td>$\alpha_2 \delta_2$</td>
<td>2.5</td>
</tr>
<tr>
<td>Fetal Hemoglobin</td>
<td>F</td>
<td>$\alpha_2 \gamma_2$</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Portland</td>
<td>$\xi_2 \gamma_2$</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Gower1</td>
<td>$\xi_2 \varepsilon_2$</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Gower2</td>
<td>$\xi_2 \varepsilon_2$</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

Under normal conditions, most of this iron is within heme, with little or none present as free metal (i.e. non-heme). Heme, a potent pro-oxidant, is in turn tightly bound to the hemoglobin molecules in a relatively redox-unavailable state. This near perfect compartmentalization of iron, however, may break down in certain pathologic states characterized by alterations in tetramer and/or globin chain stability. These pathologic states include hemoglobinopathies, such as thalassemia (19, 20). Removal of oxidatively damaged RBC $\textit{in vivo}$ occurs primarily via erythrophagocytosis by the mononuclear phagocytic system (MPS). This clearance mechanism may result in negative immunoregulatory effects on these patients, such as an increased risk of bacterial infections (21, 22).

1.3.1.1 Thalassemia Pathophysiology

The $\alpha$- and $\beta$-thalassemias are a major cause of morbidity and mortality in humans throughout the world. Thalassemia and other hemoglobinopathies are the most common genetic
disorders, affecting approximately 7% of the world population. It is estimated that 300,000-400,000 infants with severe forms of these diseases are born each year, leading to serious public health problems (23).

Thalassemia was a common condition encountered by physicians in the Mediterranean and South East Asia even before its first clinical description in 1925 (24). However, there was very little evidence that it would become a specific entity at that time. The first clinical description of thalassemia is by an American pediatrician Thomas B. Cooley, and it was clear then that the disease was caused by a different entity; Italian clinicians were also describing similar findings at about same time. The term thalassemia derives from the Greek word “thalassa,” which means “the sea” in reference to the Mediterranean, and “emia,” meaning related to blood. Whipple and Bradford first used the term “thalassemia” during 1932 in their classical paper on the pathology of conditions (25). Thalassemia is found in all parts of the world, which is why it is sometimes misleading to call the condition “Mediterranean anemia,” as it was often referred to in early days (26).

Thalassemias are the most common monogenic, genetically inherited, and heterogeneous type of blood disorders that result from over 200 different mutations of the α and β globin genes (27). In every population in which thalassemia is common, there is a different pattern of mutation, suggesting that the disease must have arisen independently in different parts of the world and then reached its high frequency due to local factors, such as drift and selection (28). Although a variety of studies suggest that the selective factor might be malaria, it is only in recent years that it has been possible to base this hypothesis on solid experimental footing. Much of this new information has come from studies of β-thalassemia in Melanesia and Polynesia (28, 29).

Thalassemias are a group of congenital blood disorders characterized by anemia caused
by the deficient synthesis of one or more of the globin chains, primarily \( \alpha \) and \( \beta \) chains. Beta-thalassemia occurs consequent to mutations or deletions in the \( \beta \) globin chain, while \( \alpha \) thalassemia arises from deletions or mutations in the \( \alpha \) globin chain. There are 4 \( \alpha \)-globin genes on chromosome 16; therefore, \( \alpha \)-thalassemia has 4 different manifestations that correlate with the number of \( \alpha \)-globin genes affected. Individuals with \( \beta \)-thalassemia are deficient in one or more of their \( \beta \)-globin genes. There are only 2 genes on chromosome 11 that code for \( \beta \)-globin chains, and therefore the incidence of \( \beta \)-thalassemia major is higher than \( \alpha \)-thalassemia major or hemoglobin H disease, because the probability of having a deficiency in 2 genes is higher than having a deficiency in 3 or 4 genes (30, 31). The primary defect in both cases is a quantitative decrease in hemoglobin; however, structural variants generating the production of unstable globin chains also give rise to thalassemic phenotypes. Thalassemic RBC show reduced or absent levels of \( \alpha_{2} \beta_{2} \) and increased concentrations of monomeric \( \alpha \)-chains and tetrameric \( \beta \)-chains (32).

There are many important studies about the pathophysiology of thalassemia conducted prior to the growth of our knowledge of the iron-mediated free radical biochemistry associated with thalassemia. However, now there is a growing body of evidence that injury to erythrocytes in thalassemia is associated with the generation of free radical species due to an excess of denatured \( \alpha \) and \( \beta \) globin chains (15). In fact, RBCs from \( \beta \)-thalassemia patients have been shown to release heme and generate a superoxide radical eight times greater than RBCs from healthy controls (33). Similarly, due to the high content of redox active iron in the cytosolic and/or membrane compartments of sickle and \( \beta \)-thalassemia RBCs, excessive hydroxyl radical formation has also been reported in these cells (34-36).

The mechanism underlying this premature destruction of red blood cells (RBC) is the release of iron atoms from the defective hemoglobin proteins (37). (Figure 1.6) The iron then
drives free radical mediated injury to other hemoglobin molecules, as well as the RBC itself (38). The dramatic effect is on RBC precursors, premature destruction in the bone marrow and extra medullary sites (39). Subsequent to this imbalance in production of globin chains, ineffective erythropoiesis, and rapid destruction of red blood cells in the bone marrow and peripheral blood, this injury causes the damaged cell to be removed from circulation thereby resulting in mild to very severe anemia. Ferrokinetic analysis reveals that in β-thalassemia patients, only 15% of $^{59}$Fe is incorporated in circulating erythrocytes, indicating that ineffective erythropoiesis could account for as much as 60 to 75% of the total erythropoiesis (40).

Figure 1.6. Damage to multiple components of the β thalassemic RBC is mediated by an iron glutathione-dependent mechanism. As thalassemic RBC circulates, haemoglobin chains autoxidize (A) giving rise to super oxide (O$_2^-$) and methemoglobin (Meth). The heme moiety of the methemoglobin chains is released, yielding free heme and heme-free globins (B). Free heme readily reacts with reduced glutathione (GSH; reaction (C)), resulting in cleavage of the heme group and the direct release of Fe$^{2+}$ (D). The free iron can then react with the ROS generated by hemoglobin oxidation and normal cellular metabolism (E), regenerating Fe$^{3+}$ which then oxidizes additional haemoglobin (F). Reactions (G) and (H) represent subsequent oxidative and non-oxidative injury to the cell. (Modified from Scott et al., 2001) (15, 41- 43)
The designations commonly used to describe β-thalassemia syndrome are based on clinical severity. The most severe form is defined as thalassemia major (TM) and characterized by transfusion-dependent anemia. Thalassemia intermedia designate a form of anemia that, independent of the genotype, does not typically require transfusion. Thalassemia minor indicates a heterozygous state, or carriers who are asymptomatic. Thalassemia minima is used in the Italian literature to indicate a carrier in whom no hematologic or clinical symptoms are recognizable; sometimes individuals with thalassemia minima are also referred as silent carriers (40).

1.3.1.2 Secondary Iron Overload

Interestingly, β-thalassemia is characterized by two distinct disease processes, one caused by nature (mutations; primary disease) that is evident in the RBC, and the second caused by our cure (blood transfusions; secondary disease) that give rise to secondary iron overload (42). However, both mechanisms are driven by iron-driven redox reactions by the same “toxin,” which is iron. Apart from thalassemia, there are many other conditions that require transfusion and lead to secondary iron overload. (Table 1.5)

Secondary iron overload can occur due to ineffective erythropoiesis, chronic liver disease, parenteral administration of iron, or ingestion of excessive amounts of iron. Thalassemia major and sideroblastic anemia are the two most studied examples of secondary iron overload resulting from regular transfusions and ineffective erythropoiesis (44). In thalassemia, abnormalities in hemoglobin synthesis can decrease the life span of normal erythrocyte. Because of severe anemia, the pools of erythrocyte precursors are markedly expanded in most hemoglobinopathy patients, and also enhance the absorption of dietary iron.

In poorly managed and untransfused patients with severe β-thalassemia major abnormally regulated iron absorption leads to an increase in iron-mediated consequences that
may, depending on the severity and demand on erythroid expansion, vary between 2-5 gram per day (45). Chronic transfusion may double this rate of iron accumulation (46). Although most clinical manifestations of iron loading are slow processes and appear in the second decade of life in inadequately chelated patients, there is evidence showing the detrimental effects of iron accumulation from liver biopsies of much younger patients (46).

Table 1.5. Causes of chronic anemia requiring multiple transfusions leading to iron overload. Modified from (47).

<table>
<thead>
<tr>
<th>Decreased RBC production</th>
<th>Hemolytic Anemia (increased RBC destruction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>○ Bone marrow aplasia (bone marrow failure)</td>
<td>○ Acquired causes</td>
</tr>
<tr>
<td>▪ Involving a single cell line (Diamond-Blackfan syndrome, or pure red cell aplasia)</td>
<td>▪ Mechanical injury to RBC</td>
</tr>
<tr>
<td>▪ Involving all cell lines</td>
<td>▪ Chronic disseminated intravascular coagulopathy</td>
</tr>
<tr>
<td>○ Aplastic anemia</td>
<td>▪ Giant hemangioma (Kasabach-Merritt syndrome)</td>
</tr>
<tr>
<td>○ Hypoplastic anemia (Fanconi anemia)</td>
<td>▪ Cardiac valve defects (usually prosthetic)</td>
</tr>
<tr>
<td>○ Myelodysplastic syndromes</td>
<td>▪ Auto immune hemolytic anemia</td>
</tr>
<tr>
<td>○ Bone marrow replacement</td>
<td>▪ Hypersplenism (secondary to splenomegaly of any cause)</td>
</tr>
<tr>
<td>▪ Leukemia (acute and chronic)</td>
<td>○ Hereditary causes</td>
</tr>
<tr>
<td>▪ Metastatic tumors spread to bone</td>
<td>▪ RBC membrane defects</td>
</tr>
<tr>
<td>▪ Myelofibrosis with myeloid metaplasia</td>
<td>▪ Acanthocytosis</td>
</tr>
<tr>
<td>○ Chronic renal disease (impaired erythropoietin production)</td>
<td>▪ Hereditary spherocytosis</td>
</tr>
<tr>
<td>○ Maintenance chemotherapy (suppression of DNA synthesis)</td>
<td>▪ Hereditary elliptocytosis</td>
</tr>
<tr>
<td>○ Dysplastic erythropoiesis (congenital dyserythropoietic anemia types I, II, and III)</td>
<td>▪ Hereditary stomatocytosis</td>
</tr>
<tr>
<td></td>
<td>▪ Hereditary xerocytosis</td>
</tr>
<tr>
<td></td>
<td>▪ Paroxysmal nocturnal hemoglobinuria</td>
</tr>
<tr>
<td></td>
<td>▪ RBC enzyme abnormalities (enzymopathies)</td>
</tr>
<tr>
<td></td>
<td>▪ Glucose-6-phosphate dehydrogenase deficiency</td>
</tr>
<tr>
<td></td>
<td>▪ Glutathione reductase</td>
</tr>
<tr>
<td></td>
<td>▪ Hexokinase</td>
</tr>
<tr>
<td></td>
<td>▪ Pyruvate kinase</td>
</tr>
<tr>
<td></td>
<td>▪ Defects of hemoglobin molecule (hemoglobinopathies)</td>
</tr>
<tr>
<td></td>
<td>▪ Hemoglobin C disorder</td>
</tr>
<tr>
<td></td>
<td>▪ Hemoglobin S-C disorder</td>
</tr>
<tr>
<td></td>
<td>▪ Hemoglobin S-S disorder (sickle cell anemia)</td>
</tr>
<tr>
<td></td>
<td>▪ β-thalassemia</td>
</tr>
<tr>
<td>Chronic blood loss</td>
<td></td>
</tr>
<tr>
<td>○ Occult bleeding (usually via the gastrointestinal tract)</td>
<td></td>
</tr>
<tr>
<td>○ Idiopathic pulmonary hemosiderosis</td>
<td></td>
</tr>
</tbody>
</table>
After approximately one year of regular transfusion therapy, iron is deposited in parenchymal tissues such as liver, kidney and heart, where it may cause significant toxicity compared to that in reticuloendothelial cells (48). As iron accumulation progresses, the major iron carrier protein transferrin reaches its full capacity to detoxify and bind the excess iron. Therefore, the non-bound iron fraction in the plasma may promote the generation of reactive oxygen species, including hydroxyl radical, which further damages tissues and cells (49). Each unit of RBC transfusion contains approximately 200-250 mg of iron (50). Transfusion-dependent patients, like thalassemia majors on hypertransfusion therapy, have an excess of iron of approximately 0.4 to 0.5 mg/kg/day, and about 1 g per month. These patients usually start to see the signs of iron overload after anywhere between 10 and 20 transfusions (46, 51).

### 1.3.1.3 Other Disorders Characterized by Iron Accumulation

Hemochromatosis describes conditions where there is increased storage and absorption of iron, leading to pathological changes (52). Hemochromatosis is divided into two major categories: primary (or genetic) hemochromatosis and secondary hemochromatosis (53). Hereditary hemochromatosis (HH) is the most common autosomal recessive disease of primarily Caucasian origin and is generally considered to be a disorder in which the increase in iron absorption and storage causes mild to severe pathologic changes resulting in fatality (54). The homeostasis of iron is strictly controlled and regulated by a major histocompatibility (MHC) class I-like molecule designated HFE. HH mutation at gene HFE results in excessive iron absorption and storage (55).

In other conditions, such as non-hemochromatoic liver disorders like porphyria cutanea tarda and chronic hepatitis, iron also plays a pathogenic role (56). Furthermore, many other
diseases which have a predisposition for iron buildup, such as Sub-Saharan African type hemochromatosis or Bantu hemosiderosis, are caused by drinking alcoholic beverages fermented in iron utensils (57). Different transfusion-dependent hemolytic anemias, including β-thalassemia major and sickle cell disease, are common conditions found in various ethnic populations. It is apparent that in all of these conditions, it is not always the primary pathology, which leads to dysfunctional cells, but rather the secondary iron overload that causes cellular injury and death.

1.4 Iron Metabolism in Normal Cells

1.4.1 Iron Absorption From the Gut and Up Take in Cells

Mammalian iron regulation is tightly controlled and well-balanced, daily absorption and loss is roughly equal, so that theoretically excess iron does not accumulate. The absorption of dietary iron takes place in the microvilli of the small intestine, where enterocytes take up iron in two forms, inorganic iron and heme. Inorganic iron (Fe$^{2+}$) is transported across the enterocyte membrane by divalent metal transporter 1 (DMT1). (Figure 7) As most non-heme iron is found to be in ferric (Fe$^{3+}$) complex state, it needs to be converted to the ferrous form to be absorbed by the cells. Although the major source of dietary iron is from heme in the human diet, the pathway of how iron is absorbed to enterocytes is not well defined. It is believed that it is either receptor mediated or that passive diffusion is involved in this process (58). At the pH of the intestine, and in other neutral parts of the body, Fe$^{3+}$ ions form large insoluble complexes of iron and oxygen; whereas most of the other ions, including ferrous iron, are stable as solitary ions surrounded by water (59, 60).

The primary function of iron in the body is to transport oxygen. Hemoglobin, which is present in red blood cells, is essential for transferring oxygen from the lungs, via blood, to the
tissues; myoglobin is present in muscle cells and accepts, stores, transports, and releases oxygen in the muscles. About six percent of body iron is used as a component of certain proteins (it forms part of the cytochromes, which are essential for respiration and energy metabolism); as a component of certain enzymes, iron is involved in the synthesis of collagen and various neurotransmitters. In addition, iron is required for proper immune function (17).

**Figure 1.7. Scheme of iron absorption and transport across the enterocyte.** Iron is absorbed into the cell through an active transport process involving the protein DMT-1 and Heme transporter. Iron may leave the enterocyte and enter blood circulation via the basolateral transporter known as ferroportin. It can be bound to ferritin, an intracellular iron-binding protein transferrin-1. Iron is stored in ferritin and remains in cells. This iron will be lost from the body when the enterocyte dies and is sloughed off from the tip of the villus. Iron that enters the blood from the basolateral surface of the enterocyte is bound to transferrin, delivers iron to red blood cell precursors in the bone marrow, that take up iron bound to transferrin via receptor-mediated endocytosis. Figure is original artwork (© Mustafa, 2011) (61).

### 1.4.2 Distribution of Bodily Stores of Iron

The divalent ferrous form (Fe$^{2+}$) and trivalent ferric form (Fe$^{3+}$) of iron are the two most important types present in most biological systems, because both forms can donate and accept electrons which makes them crucially important in many enzymatic reactions (62). Only 300 mg of iron are present in humans at birth, the remainder being accumulated over the life span (63).
The average amount of iron in healthy adult males and females has been estimated to be 4 to 5 grams respectively (63). The level of both functional iron and the storage of iron are principally controlled by its absorption into the upper portions of the small intestine; there is no currently known excretory mechanism for excess iron in the body (64). (Figure 1.8)

**Figure 1.8. Distribution and compartmentalization of iron in the body.** Iron distribution in normal adult humans in a balanced state is in different compartments of the body. Normally, from diet, 1-2 mg of iron enters and leaves the body every day, either by the sloughing of cells or desquamation; there is no well-defined excretory pathway. Dietary iron is absorbed by duodenal entrecotes. The iron circulates in the plasma with the carrier protein transferrin. Most of the iron in the human body is incorporated into hemoglobin in erythroid precursors in the bone marrow and mature erythrocytes. Iron is stored in parenchymal cells of the liver and reticuloendothelial macrophage system, including bone marrow and the spleen. These macrophage systems provide most of the useable iron by degrading hemoglobin from senescent RBC and reloading iron for further delivery to the cell. Figure is original artwork (© Mustafa, 2010) (65, 66).
1.4.3 Biological “Detoxification”/Buffering of Misplaced/Excess Iron

Metal ions and their interactions in biological systems depend to a large extent on the chemical form of the metal ion in biological fluids, because the formation of metal complexes is highly favored thermodynamically. Most metals are present in biological tissues and fluids as complexes, rather than free cations. Metal detoxification or protection from toxicity usually involves binding to specific proteins, including metallothioneins, which form complexes with copper, zinc, cadmium, mercury, and other metals. Iron-binding proteins are, predominantly, ferritin, transferrin and hemosiderin, but they also have some affinity for other metals (67).

1.4.3.1 Transferrin

Physiologically, the majority of cells in organisms acquire iron from the well-characterized plasma glycoprotein transferrin, approximately 80 kDa (68, 69). Transferrin contains two homologous domains, each of which contains one high affinity Fe\(^{3+}\)-binding site. The affinity of iron to transferrin is a pH dependent process. In plasma with pH 7.4, iron binds very strongly to transferrin (Kd approximately $10^{-23}$ mol/liter), whereas virtually no binding occurs at pH $\leq$4.5; this property plays an important role in the mechanism of iron release from transferrin. Iron is transported in plasma as a deferred transferrin complex, and under normal physiological conditions, transferrin is in excess of iron (60,70, 71). However, a significant concentration of non transferrin-bound “free” or “misplaced” iron has been reported in iron-overload disorders with fully saturated transferrins, whereas non transferrin-bound iron is not present in healthy controls. The concept that transferrin-bound iron is taken to cells, by membrane-specific receptors, was proposed by Jandl and Katz in 1963. It is now a well-known and well-characterized phenomenon in cell function and the regulation of iron.
The structure of the human transferrin receptor has a disulphide-linked dimer of two identical transmembrane subunits, both of approximately 90 kDa and containing 760 amino acid residues (72). These amino acid residues enable ligand binding to the transferrin, as each monomer can bind to one or two molecules of iron. The interaction of transferrin and its receptor is reversible, pH dependent, and influenced by its iron content. At a near neutral pH, the receptor has a higher affinity for diferric transferrin than for apotransferrin, in addition to an intermediate affinity for monoferric transferrin (73).

Normally, the plasma iron concentration is 10-30 μM/l (mean about 20 μM/l) and the plasma transferrin concentration is 22-35 μM/l (mean 30 μM/l) so that the transferrin is about 30% saturated with iron (range-20-50%) (74). Thus, the plasma and intestinal fluid contain a considerable amount of iron-free transferrin, which is able to bind iron absorbed from the intestine or iron released from cells.

1.4.3.2 Ferritin

Ferritin is a ubiquitous protein with only one clear function; the sequestration and storage of iron. The ferritin molecule has an important role in the overall physiology of iron metabolism: (i) its ability to maintain iron in a soluble, non-toxic, and biologically useful form, and (ii) its capacity to sequester vast quantities of iron. Ferritin is composed of an appoprotein (MW, approximately 480 kDa) that surrounds a core of up to 4,500 atoms of iron in the form of ferricydrite (75). The ferritin molecule is composed of 24 subunits of two structurally distinct subunit types. The heavy or H-subunit has a more acidic pH and a molecular weight of approximately 21 kDa. The light or L-subunits are more basic than the H subunit and have a molecular weight of approximately 19 kDa (76). About 25 percent of the
iron in the body is stored as ferritin, which is found in cells and circulates in the blood (77). The average adult has serum ferritin; male 15-200 ng/ml and female 12-150 ng/ml (78).

1.4.3.3 Hemosiderin

Hemosiderin, another iron-storage complex, is a water insoluble degradation product of ferritin, which appears to result from incomplete lysosomal processing. Its molecular nature remains poorly defined, but it is always found within cells (as opposed to circulating in blood) and appears to be a complex of ferritin, denatured ferritin, and other material. The iron within deposits of hemosiderin is not readily available to supply iron when needed. Hemosiderin is most commonly found in macrophages and is especially abundant in situations following hemorrhage, suggesting that its formation may be related to phagocytosis of red blood cells and hemoglobin (77, 79).

1.5 Why is “Misplaced” Iron Dangerous?

1.5.1 Iron Chemistry and Biochemistry

Iron is a chemical element with the symbol Fe and atomic number 26. It is a metal in the first transition series. Like other Group 8 elements, iron exists in a wide range of oxidation states, from -2 to +6, although +2 and +3 are the most common. All the metals in the first row of the d block in the Periodic table contain unpaired electrons, and can thus qualify as radicals, with the sole exception of zinc. The melting point of iron is 1535 °C, the boiling point is 2750 °C, and the specific gravity is 7.874 (20 °C), with a valence of 2, 3, 4, or 6. Pure iron is chemically reactive and corrodes rapidly, especially in moist air or at elevated temperatures (80).
Iron is one of the major ubiquitous metallic elements in cells, present in the structures of many enzymes and proteins. Indeed, iron cores constitute the functional sites of many enzymes and proteins involved in generating energy, transporting oxygen, and synthesizing DNA. Iron is a vital trace element for all living cells. The functions of this important metal are vital; while this metal is important, maintaining its biological balance in an organism is far more crucial than virtually any other trace element (with the possible exception of copper) (81, 82).

The versatility of uses that nature has found for iron originates in the simple aqueous chemistry of this essential transition metal. As a transition element, iron has the potential to produce a variety of reactions where one electron transfer is significant. In point of fact, it is this important factor, the “electron transfer” attribute, that makes iron useful for enzymatic catalytic redox reactions (83). Although iron is biologically imperative as a component of enzymes, free iron has particularly harmful consequences due to free radical reactions. Iron accumulation and the presence of free iron within cells facilitate hydroxyl radical generation and adverse oxidation reactions (84). Indeed, numerous studies have clearly demonstrated that cells and organisms cannot tolerate excess iron. Despite its potential toxicity, normal physiology has no mechanism for iron excretion from the body, except for that associated with the desquamation of skin cells or bleeding (45).

Iron is recognized as a potent pro-oxidant and necessary catalyst for *in vivo* lipid peroxidation, cellular anabolism, and catabolism. *(Table 1.6)* Organ damage arising from chronic iron overload is remarkable for the range of tissues affected (both explicative and non-explicative) and for the slow and insidious onset of organ dysfunction. Foremost amongst the organs and cell types affected by iron overload are the liver, heart, and
pancreatic beta cells. Given the tendency of transition metals, such as iron, to amplify oxidant damage, organs with very active mitochondria are targeted in iron overload disorders (85). (Figure 1.9)

Figure 1.9. Scheme representing iron uptake and mechanism of iron-mediated cellular injury. (A) Transferrin-bound iron is transported into the cell by receptor-mediated endocytosis. HFE gene represents the transferrin receptor (TR), where mutations at HFE increase intestinal absorption of iron. (B) Iron is released from transferrin by change in pH; enters the intracellular labile iron pool. (C) Iron is stored in the cells as hemosiderin and ferritin. (D) Once iron is released, transferrin releases by exocytosis. (E) The iron metabolism is a vital part of cells, and most of the iron is in hemoglobin, during which the metabolism and catabolism of cells ROS are formed. These radicals directly damage mitochondria and decrease the function of active mitochondria. (F) As a result of ROS, lipid peroxidation takes place, leading to the formation of aldehydes, which damage nuclear membranes and DNA. This damage leads to mutations and dysfunctional cells, generating different diseases, such as diabetes and cancer. (G) DNA damage affects transcription and protein synthesis, which leads to altered apoptosis and the aging of cells. (H) Where iron accumulates increases, transferrin levels also increase. Figure is original artwork (© Mustafa, 2010) (45, 86, 87).
1.5.2 Redox Chemistry and Toxicity of Iron

In normal aerobic metabolism, oxygen acts as a terminal electron acceptor. Even though O\textsubscript{2} is not directly toxic to organisms, it can be unstable due to the presence of 2 half-filled outer orbitals. As a result of this configuration, O\textsubscript{2} undergoes four one-electron reductions to water (H\textsubscript{2}O) and CO\textsubscript{2}. The immediate reduction products, superoxide (O\textsubscript{2}\textsuperscript{-}), and hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) are the underlying agents causing toxicity of O\textsubscript{2}. The superoxide, as a reducing agent, readily reacts with iron or copper. The one electron reduction of O\textsubscript{2} by Fe\textsuperscript{2+} results in O\textsubscript{2}\textsuperscript{-} formation, which in turn leads to a sequence generating a hydroxyl radical as shown in Figure 1.10 (88).

These many events contribute to the oxidation-reduction process known as Haber-Weiss, also associated with Fenton reactions (89). Iron accumulation and the quantity of iron becomes the constant rate for these oxidation reactions that generate highly reactive hydroxyl radicals. In normal physiology, there is no mechanism for iron excretion from the body except for a negligible amount by desquamation of iron-laden macrophage cells. Without any iron excretory pathway, an iron rich diet also contributes to increased iron absorption and the build-up of non-utilized iron. Many organisms cannot tolerate the excess iron and the invariable self-propagating iron-driven cellular injuries (90).

\[
\begin{align*}
\text{Fe}^{3+} + \text{O}_2\textsuperscript{-} & \rightarrow \text{Fe}^{2+} + \text{O}_2 & (1) \\
\text{Fe}^{2+} + \text{H}_2\text{O}_2 & \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^- & (2) \\
\text{Net}: \text{O}_2\textsuperscript{-} + \text{H}_2\text{O}_2 & \xrightarrow[\text{Catalyst}]{\text{Fe}} \text{O}_2 + \text{OH}^- + \text{OH}^- & (3)
\end{align*}
\]

**Figure 1.10. Iron-catalyzed redox reactions of biological importance.** Equation 1 shows the reduction of Fe\textsuperscript{3+} by the superoxide radical (O\textsubscript{2}\textsuperscript{-}). Equation 2 is the Fenton reaction that results in the production of the hydroxyl radical (OH) and the hydroxide anion (OH\textsuperscript{-}) from hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}). Equation 3 shows the iron-catalyzed Haber-Weiss reaction (90).
Table 1.6. The effect of iron on cell organelles. (91)

<table>
<thead>
<tr>
<th>Sites</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysosomes</td>
<td>Lipid peroxidation, increased volume, increased fragility, decreased membrane fluidity and increased pH</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Lipid peroxidation, impaired oxidative metabolism, decreased calcium sequestration, increased calcium release</td>
</tr>
<tr>
<td>Endoplasmic reticulum</td>
<td>Lipid peroxidation, decreased cytochrome P-450 levels, decreased calcium sequestrations</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>Altered lipid composition</td>
</tr>
<tr>
<td>Nucleus</td>
<td>Increased DNA strand breaks</td>
</tr>
</tbody>
</table>

1.5.2.1 Consequences of Misplaced/Excess Iron in Transfusion Medicine

Although the transfusion of RBC saves the lives of transfusion-dependent patients, transfusions are not without risk. RBC transfusions with prolonged storage are associated with increased morbidity and mortality. The exact mechanism remains unknown, but there is evidence in animal studies that transfusion with prolonged-storage blood has iron-mediated consequences (92). Observational studies suggest that prolonged RBC storage before transfusion increases mortality, serious infection, and multi-organ failure in some hospitalized patients. These findings suggest there are immune modulatory effects due to large amounts of iron in monocytes /macrophages (93). For example, in hemochromatosis, macrophages have decreased intracellular iron levels, which results in decreased cytokine production. Conversely, increased intracellular iron can exacerbate the systemic inflammatory response syndrome, which can lead to deleterious consequences (94). Also, increased circulating iron, especially non transferrin-bound iron (NTBI), enhances the proliferation of certain pathogens. Red blood cell transfusion increases NTBI levels in neonates and may increase NTBI levels in patients with thalassemia; however, studies of other patient populations have not been reported (95-97).
1.5.2.2 Iron and the Immunological Status of Thalassemia

Apart from the primary pathology of thalassemia, there is an imbalance in hemoglobin leading to the premature destruction of RBC and severe hemolytic anemia. In addition, it is well documented that further complications arise from iron overload as a secondary manifestation of the disease (98). Another important clinical observation is that thalassemic patients are prone to recurring common bacterial infections (99). (Table 1.7) Studies of immune cell function in clinical situations of iron overload emerged due to interest in the greater susceptibility to infections found in splenectomised thalassemia major patients (100). While much of the data relating to increased susceptibility to infection dates from the 1960’s to early 80’s, a confounding variable for some immunological studies may be the appearance of HIV infection in thalassemia patients in the late 1980’s. HIV infection became more commonplace in thalassemia major patients as viral blood screening was not mandatory, or even possible, for HIV in many Asian countries (101, 102).

Table 1.7. Common recurrent bacterial infections in thalassemia patients.

<table>
<thead>
<tr>
<th>Klebsiella pneumoniae</th>
<th>Aeromonas hydrophila</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>Staphylococci spp</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>Streptococcus pneumoniae</td>
</tr>
<tr>
<td>Enterobacter spp</td>
<td>Yersenia enterocolitica</td>
</tr>
<tr>
<td>Acenatobacter spp</td>
<td></td>
</tr>
</tbody>
</table>

There are numerous reports of immune abnormalities reported in thalassemia major patients. Some of the important immunological markers are either up regulated or down regulated. The ratio of T lymphocytes CD4/CD8 proliferation is decreased. (Table 1.8) Other immune markers, such as complement components C3 and C4, and natural killer cell functions were defective. The factors, which cause these alterations, are not very well defined, but it is very likely that iron overload is the major factor that contributes to these
abnormalities (103). The suppression of cytokines interleukin 2 (IL-2), interferon gamma (INF-γ), and IL-4 has also been reported. Patients with high ferritin produce significantly less INF-γ and IL-2, indicating that immunosuppression observed in thalassemia major is an effect of iron overload (93, 104).

Table 1.8. Immunological parameters in β-thalassemia major patients and healthy individuals. Data modified from (93)

<table>
<thead>
<tr>
<th>Cells</th>
<th>Patients (n = 28)</th>
<th>Controls (n = 30)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>3,248.8 ± 1,668.4</td>
<td>2,255.3 ± 561.3</td>
<td>0.003</td>
</tr>
<tr>
<td>CD3+</td>
<td>2,462.7 ± 1,142.4</td>
<td>1,478.5 ± 408.5</td>
<td>0.001</td>
</tr>
<tr>
<td>CD19+</td>
<td>302.9 ± 218.1</td>
<td>158.1 ± 118.6</td>
<td>NS</td>
</tr>
<tr>
<td>CD4+</td>
<td>1,544.8 ± 776.1</td>
<td>967.0 ± 236.2</td>
<td>0.001</td>
</tr>
<tr>
<td>CD8+</td>
<td>832.4 ± 453.8</td>
<td>725.6 ± 287.9</td>
<td>NS</td>
</tr>
<tr>
<td>CD4+/CD8+</td>
<td>1.8 ± 0.8</td>
<td>1.4 ± 0.6</td>
<td>0.038</td>
</tr>
<tr>
<td>CD3+/CD16+CD56+</td>
<td>140.1 ± 131.7</td>
<td>106.7 ± 90.5</td>
<td>NS</td>
</tr>
<tr>
<td><strong>T-cell activation markers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3+/HLA-DR+</td>
<td>164.9 ± 108.7</td>
<td>71.7 ± 40.3</td>
<td>0.000</td>
</tr>
<tr>
<td>CD3+/HLA-DR+ (%)</td>
<td>5.02 ± 3.2</td>
<td>3.2 ± 1.4</td>
<td>0.036</td>
</tr>
<tr>
<td>CD3+/CD25+</td>
<td>248.7 ± 143.4</td>
<td>105.6 ± 52.9</td>
<td>0.000</td>
</tr>
<tr>
<td>CD3+/CD71+</td>
<td>50.3 ± 46.7</td>
<td>19.1 ± 10.6</td>
<td>0.041</td>
</tr>
<tr>
<td><strong>T-cell proliferation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stimulation index (SI)</td>
<td>4.1 ± 1.8</td>
<td>5.1 ± 1.7</td>
<td>0.033</td>
</tr>
<tr>
<td><strong>Cytokines/neopterin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neopterin (nmol/l)</td>
<td>5.5 ± 2.1</td>
<td>4.3 ± 0.7</td>
<td>0.011</td>
</tr>
<tr>
<td>IFN-γ (pg/ml)</td>
<td>555.5 ± 441.2</td>
<td>893.6 ± 457.3</td>
<td>0.013</td>
</tr>
<tr>
<td>IL-4 (pg/ml)</td>
<td>21.0 ± 14.3</td>
<td>74.4 ± 43.4</td>
<td>0.000</td>
</tr>
<tr>
<td>IL-2 (pg/ml)</td>
<td>1,840.0 ± 1012.6</td>
<td>2,403.4 ± 632.1</td>
<td>0.000</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>6.6 ± 6.8</td>
<td>5.7 ± 4.6</td>
<td>NS</td>
</tr>
</tbody>
</table>

Results are expressed as the mean ± SD. Values for cells are in absolute cell count per microliters and percentage. P value<0.05 considered significant in comparison with control. NS Not significant

In thalassemia, the mechanism underlying this premature destruction of RBC is the release of iron atoms from defective hemoglobin proteins. The iron then drives free radical mediated injury to other hemoglobin molecules, as well as the RBC itself. This injury, in turn, causes the damaged cell to be removed from circulation, thereby resulting in the observed anemia. Removal of oxidatively damaged RBC in vivo occurs primarily
via erythrophagocytosis by the mononuclear phagocytic system (MPS) (105). (Figure 1.11) This clearance mechanism may result in negative immunoregulatory effects, such as an increased risk of bacterial infections in these patients.

**Figure 1.11.** Erythrophagocytosis of abnormal erythrocytes by mononuclear phagocytic system (MPS) may lead to immune dysfunctions by modulating various components in the system. Abnormal erythrocytes, such as thalassemic cells (A) circulate excessive α-hemoglobin chains that may involve the auto-oxidation of hemoglobin chains (B) releasing free heme (C) and free iron (D) in such excess that MPS cannot pedal the normal physiology, which could lead to altered phagocytosis. The cell recognition and surface CD markers may also be damaged or weakened by excessive iron recycling (E); as a result, there will be impaired immune response and T cell proliferation (F), in addition to the detrimental effect on cytokines (G), which mediate antigen processing and signaling pathways. Figure is original artwork (© Mustafa, 2010)

1.6 Iron Chelation in Transfusion Medicine

1.6.1 History of Iron Chelation

Since Dr. Cooley came up with the first clinical description of thalassemia, clinicians and scientists have done a wide range of work to understand the molecular basis, pathophysiology, and management of thalassemia syndrome. Prior to developments in
modern blood banking technologies, most seriously afflicted individuals (e.g.; β-thalassemia major) died early in life, typically after the hemoglobin synthesis switched from fetal hemoglobin (HbF) to adult hemoglobin (HbA). (Figure 1.12) Most of the patients died in the early teenage years from cardiac complications. However, the introduction of reliable sources of RBC as blood component in transfusion services from the late 1940s and throughout the 1950s resulted in the implementation of hypertransfusion of thalassemic patients to correct life-threatening severe hemolytic anemia. While thalassemia patients were treated with regular blood transfusions, the survival beyond early teens were disappointing. Early in 1960, Deferoxamine (DFO), the first iron chelation treatment, was introduced to thalassemia patients (106-108).

Figure 1.12. Survival in transfusion-dependent β-thalassemia majors. Advances in hypertransfusion and chelation therapy have yielded significant improvements in the life span and quality of life of individuals with severe thalassemia. While hypertransfusion therapy alone prolonged survival into the second and third decade of life, it was only with the introduction of chelation therapy that modern management of thalassemia was achieved. (Modified from Scott 2001) (42, 109)
1.6.2 Current and Potential Iron Chelators

The most commonly used iron chelator for the past four decades is a hexadentate iron chelator, deferroxamine, and still it is the gold standard for iron chelation therapy. However, most of the newly developed iron chelators are bidentate or tridentate due to difficulties in oral bioavailability of hexadentate chelators, such as DFO (110). Iron has a coordination number of six, and the central iron atom coordinates six monodentate ligands to form an octahedral chelate. Ligands where more than one atom may be coordinated are termed bidentate, tridentate, or hexadentate (111).

Even though DFO is proven to be effective in iron chelation, not all of the patients were willing to cope with the painstaking requirement of long-term subcutaneous injections; most use syringes, only a few use pumps. In addition, the high cost of DFO is a serious obstacle for many patients who require the treatment. In view of these problems, there is a great need for a cost effective alternative iron chelator to DFO, a concern that has been at the forefront of iron chelation research. In recent years, many iron-chelating agents were tested and some of them have continued into animal studies and first phase human clinical trials. The most commonly used iron chelators in clinical practice are desferrioxamine (Desferal®), deferiprone (L1, Ferriprox®), and Deferisirox (ICL670, Exjade®) (112-114). (Table 1.9) Figure 1.14.
Table 1.9. Characteristics of commonly used iron chelators in clinical practice (48,112, 115 -119)

<table>
<thead>
<tr>
<th>Features</th>
<th>Deferoxamine</th>
<th>Deferiprone</th>
<th>Deferasirox</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denticity</td>
<td>Hexadentate</td>
<td>Bidentate</td>
<td>Tridentate</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>560</td>
<td>139</td>
<td>373</td>
</tr>
<tr>
<td>MW Complex with Iron</td>
<td>619</td>
<td>470</td>
<td>798</td>
</tr>
<tr>
<td>Protein Binding</td>
<td>&lt;10%</td>
<td>&lt;10%</td>
<td>99%</td>
</tr>
<tr>
<td>Partition Coefficient</td>
<td>0.02</td>
<td>0.18</td>
<td>6.3</td>
</tr>
<tr>
<td>Charge</td>
<td>Positive</td>
<td>Neutral</td>
<td>Negative</td>
</tr>
<tr>
<td>Iron: Chelator Complex</td>
<td>1:1</td>
<td>1:3</td>
<td>1:2</td>
</tr>
<tr>
<td>Plasma Clearance Time</td>
<td>20 minutes</td>
<td>53-166 minutes</td>
<td>1-16 hours</td>
</tr>
<tr>
<td>Oral Absorption</td>
<td>Negligible</td>
<td>Peak 45 minutes</td>
<td>Peak 1-2.9 hours</td>
</tr>
<tr>
<td>Route of Excretion</td>
<td>Urinary, Fecal</td>
<td>Urinary</td>
<td>Fecal</td>
</tr>
<tr>
<td>Usual Dose mg/kg/day</td>
<td>25-60</td>
<td>75</td>
<td>20-30</td>
</tr>
<tr>
<td>Route of Administration</td>
<td>Subcutaneous or intravenous 8-12 hours, 5 days/week</td>
<td>Oral 3 times daily</td>
<td>Oral once daily</td>
</tr>
<tr>
<td>Membrane Permeability</td>
<td>Minimal and slow</td>
<td>Readily absorbed in gut</td>
<td>Permeates membrane</td>
</tr>
<tr>
<td>Clinical Experience since</td>
<td>1962</td>
<td>1987</td>
<td>2005</td>
</tr>
<tr>
<td>Trade Name</td>
<td>Desferal®</td>
<td>Ferriprox®</td>
<td>Exjade®</td>
</tr>
<tr>
<td>Side Effects</td>
<td>Ototoxicity Retinal Toxicity Growth Cartilage</td>
<td>Agranulocytosis Arthropathy Gastrointestinal disturbances Transient transaminits Zinc deficiency</td>
<td>Gastrointestinal disturbances, mild non-progressive creatinine increase, elevated liver enzymes, ocular and auditory abnormalities.</td>
</tr>
<tr>
<td>Status</td>
<td>Licensed</td>
<td>Not licensed in USA/Canada</td>
<td>Licensed</td>
</tr>
</tbody>
</table>

1.6.2.1 Deferoxamine (DFO)

Deferoxamine (desferrioxamine B) is derived from ferrioxamine B, a sideramine isolated from *Streptomyces pilosus* in 1960. (Figure 1.13) It has a high binding affinity for trivalent iron (Fe³⁺), which can be exploited to clinically remove excess iron from blood and tissue (107). Deferoxamine is available for patients by its trade name Desferal® (Deferoxamine mesylate USP); initially manufactured by Ciba Geigy in Switzerland, Novartis and its companies around the world have manufactured the medication since 1996 (120).
Currently, DFO is used for the treatment of acute iron poisoning and secondary iron overload conditions, such as thalassemia major, in addition to aluminum poisoning associated with chronic renal dialysis (121, 122). The goal of DFO treatment is to prevent iron accumulation in parenchymal tissues and organs that will otherwise lead to cellular injury and organ failures due to iron accumulation and iron mediated toxicity. Early treatment prior to distribution of iron into the tissue and the reticuloendothelial system has long been thought to be the most effective application of the treatment (123).

DFO has been the gold standard iron chelator since the 1970s. DFO is both safe and effective for transfusional iron overload. A hexadentate chelator, it binds iron tightly, and the iron-DFO complex is excreted in both urine and stool. DFO is administered as long parenteral infusions subcutaneously because the plasma half-life is short (~5-20 minutes), and it is not active orally. Thus, the usual dosage of DFO is subcutaneous or intravenous infusion for 8-12 hours of 20-60 mg/kg/day, 5-days/ week. The DFO-iron complex is charged and does not readily enter and leave cells. Parenteral administration and the daily routines of an infusion pump hinder the best possible form of compliance, especially for younger patients. Over the past four decades, DFO has had an extraordinary effect on the
survival rate of thalassemia patients throughout the world. Deferoxamine has intrinsic
toxicity, subacute effects consisting of nephrotoxicity, ototoxicity, and retinal toxicity have
been reported. These effects generally appear following long-term administration for chronic
iron overload; however, nephrotoxicity has been reported in patients treated for acute iron
poisoning (45, 124).

1.6.2.2 Deferiprone (L1)

Deferiprone (L1, Ferriprox®) is an orally active hydroxypyridineone first used on
humans in 1987. (Figure 1.14) Deferiprone is a bidentate chelator (3 molecules surround one
iron atom). An advantage of this compound is that the iron (III) chelate of deferiprone carries
no net charge and therefore can penetrate membranes easily, allowing the removal of
potentially toxic iron from tissues. During the 1990s, controversy about the safety of
Deferiprone was raised by Dr. Nancy Oliveri, because of her observations of liver fibrosis in
some clinical trial studies (125). However, in subsequent studies this problem has not been a
significant toxicity issue for deferiprone. In India, it is also manufactured by an Indian
company, Cipla, and is available as capsules under the name Kelfer®. Deferiprone often
causes gastrointestinal symptoms. Idiosyncratic side effects that are potentially severe include
erosive arthritis (common in patients in South Asian countries, from 5% to >20%) and
neutropenia (up to 5% of patients), comprising severe agranulocytosis (up to 0.5% of
patients); as a result, close monitoring is required. Typical dosage for deferiprone is 75
mg/kg/d in 3 divided doses, and up to 100 mg/kg daily. Deferiprone is available as an oral
capsule by different trade names in Europe and Asia, including Feriprox® and Kelfer® (116,
118, 126-129).
1.6.2.3 Deferasirox (ICL670)

Deferasirox (ICL-670; Exjade®) represents the new class of tridentate iron chelators with a high specificity for iron. The drug is FDA approved, and is an oral iron chelator developed by Novartis, the same company that manufactures Deferoxamine. It is an N-substituted bis-hydroxyphenyl-triazole, that was selected from more than 700 compounds as part of a rational drug development program. (Figure 1.15) (45, 131). Two molecules of the chelator are required to form a complete complex with ferric iron. Deferasirox chelates iron both from the reticuloendothelial cells (RE cells), as well as various parenchymal organs; the chelated iron is then cleared by the liver and excreted through bile (132). With a plasma half-life of 8 to 16 hours, once-daily dosing permits the circulation of the drug at all times to scavenge non transferrin-bound labile plasma iron, the chemical species responsible for tissue damage in iron-overloaded subjects, by means of toxic oxygen intermediaries. Exjade® tablets for oral suspension contain 125 mg, 250 mg, or 500 mg of deferasirox (117). There are reports of toxicity issues with deferasirox, including acute renal failure and some cases of Fanconi syndrome (133).
1.6.2.4 N,N-bis(2-hydroxybenzyl)ethylenediamine-N,N-diacetic acid (HBED)

HBED is a synthetic hexadentate ligand that, like DFO, forms a 1:1 complex with iron with high affinity and selectivity. It is a phenolic aminocarboxylate iron chelator, and has also been tested by a range of relevant studies, which demonstrate its effectiveness in transfusional tissue (liver) iron overload (134). HBED is significantly more effective than DFO when given intra-muscularly to iron overloaded rats. It binds ferric iron strongly with an overall log stability of constant 40, rendering this molecule a potent ligand for chelation of iron \textit{in vivo}. Unfortunately, HBED is not efficiently absorbed via the oral route in primates or humans because of the zwitteronic nature of the molecule. Consequently, research has been conducted to modify HBED with ester prodrugs (135).

1.6.3 High Molecular Weight Iron Chelators

Most of the iron chelators currently available in clinical practice and some of the potential iron chelators in the research and development phase are low molecular weight iron
chelators. The toxicity and adverse effects of current metal chelators derive, in part, from the ability of these chelator compounds to diffuse rapidly into cells and chelate essential intracellular metal stores (e.g., iron). In the absence of these essential trace metals, the viability of the cell is adversely affected. Between the loss of the chelator into cells and their rapid clearance via the kidney, the vascular retention time of current chelators is also very poor. The therapeutic dosage of Desferal® is sufficient to cause significant injury to the patient receiving the compound, which can be demonstrated by growth retardation, peripheral neuropathies, and, in mice, LD50s (~250 mg/kg) only slightly above those of the typical therapeutic dosage (20-60 mg/kg) in humans (136, 137). In the USA, on the basis of 2006 wholesale purchase prices, the annual costs of deferoxamine for ICT have been estimated to range from $6,824 to $29,209, plus $9,286 for infusion, and the estimated annual costs of deferasirox range from $24,404 to $53,095, with the actual cost depending on dose and body weight (138).

While DFO has enjoyed over four decades of clinical experience, important therapeutic applications, and high demand, it is beleaguered by many disadvantages, the most significant of which is its high toxicity and very short plasma half-life (~5.5 minutes) (139). Other problems include the drug’s high manufacturing costs, since it is a bacterial siderophore, and the hydrophilicity of the ligand resulting in it being poorly absorbed into the gastrointestinal tract. As a result of these problems, more than 30 years of research for alternatives to low molecular weight chelators and, to a lesser extent, high molecular weight chelators, has been conducted. While low molecular weight chelators demonstrate some toxicity near therapeutic dosing, the rather limited work concerning high molecular weight chelators is very promising (140).
Some of the initial modulations of DFO with biocompatible polymers were reported by Hallway et al., 1989 (137). This study showed the synthesis of high molecular weight iron chelators by covalently attaching DFO to biocompatible polymers, such as dextran and hydroxyl ethyl starch (HES). These DFO-polymer conjugates not only provided longer plasma half-lives, but also lowered the toxicity while maintaining the same iron chelation capacity as the free DFO monomer or the drug. The iron binding properties of DFO were virtually unchanged after attachment procedures, but toxicity and circulatory half-life were remarkably reduced. In addition, it has proven to be effective in inhibiting iron-driven lipid peroxidation comparable to the DFO monomer. Animal studies have shown that the LD50 in mice, based on DFO equivalent, is ~4000 mg/kg for dextran-DFO in comparison to 250 mg/kg for free DFO. HES-DFO increased plasma half-life more than 10 fold more than dextran-DFO (137, 141).

In the first reported human trial, chelator HES-DFO was administered to healthy male subjects by intravenous infusion over a 4-hour period. The drug was well tolerated, and signs of DFO acute toxicity were not observed. Maximum plasma chelator levels of approximately 3 M/l were achieved with HES-DFO, which is a plasma chelator level more than an order of magnitude higher than has been reported with injections of DFO. Drug residence time in plasma was markedly prolonged, with an initial half-life of 22 to 33 hours. Urinary iron excretion was 7.1 ± 2.2 mg in 48 hours in the highest dose group, compared with 0.06 ± 0.15 mg in control subjects who received normal saline infusions. In summation, intravenous infusion of HES-DFO is well tolerated, produces substantial and prolonged plasma chelator levels, and markedly stimulates urinary iron excretion (142).
In 2006 it was reported that a clinical trial was conducted using starch conjugated to DFO (S-DFO; 40SD02) (Figure 1.17), a high molecular weight chelator possessing prolonged vascular retention time. This chelator was manufactured by Biomedical Frontiers, Inc., Minneapolis, MN, USA, a modified version of the initial HMW chelator HES-DFO by Hallaway et al. in 1989. The new formulation differs from HES-DFO in two important respects: first, it was designed to have a shorter circulatory half-life to prevent accumulation of high-molecular-weight fragments in the circulation, and second, the number of DFO molecules bound per unit of starch is approximately 50% greater. Single doses of S-DFO were given to transfusion-dependent β-thalassemia major patients in quantities of 150, 300, 600, and 900 mg/kg. Urinary iron excretions were evaluated, and other drug-related effects were also examined. S-DFO stimulated very significant urinary iron excretion with the largest dose (900 mg/kg) S-DFO, which makes it a highly promising and potentially therapeutic drug for the purposes of achieving iron balance in poorly compliant patients (143)

Figure 1.17. Chemical structure of Starch-DFO is polydispersed with an average molecular weight of 26000 Da. The starch backbone of the drug is susceptible to cleavage by serum α-amylase(s). Cleavage results in S-DFO fragments, which are excreted primarily via the kidney. Modified from Harmatz, 2007. (143)
1.6.3.1 Novel High Molecular Weight Iron Chelators: New Class

In 2007, a patented new class of polymer-based HMW iron chelators were invented by Kizhakkedathu and Scott and synthesized within Kizhakkedathu’s laboratory (136). Based on recent advances in the field of polymer therapeutics focused on improving the therapeutic index of drugs through the development of novel, poly (ethylene glycol) (PEG)-based drug-polymer conjugates (144, 145). In most cases, the drug is attached to the polymer via a degradable linkage, such as a hydrolysable ester bond, to ensure the release of the drug at a predetermined site of action. Once the payload has been delivered, it is essential that the polymer is then cleared from circulation (140).

In this new class of HMW chelators, PEG was used as a backbone to attach monomers of various potential metal-binding chelators (e.g., DFO, ICL-670 or HBED). (Figure 1.18) The chelators used were derivatives of extensively studied compounds from the ongoing research on thalassemia in Scott’s laboratory that utilized PEG and its derivatives. Initially, ICL-670, an oral chelator, was synthesized and attached to PEG by an acrylate backbone (SK-1). Compared solubility shows ICL-670 as a monomer is insoluble in water, but pegylation has improved its solubility. The iron binding was not affected by the conjugation of PEG to ICL-670 (136).
Figure 1.18. Chemical structures of PEG-HBED and PEG ICL-670 (SK-1) should exhibit significant utility as non-permeable high molecular weight chelators. PEG-acrylate is used as a non-immunological carrier backbone to which either HBED or ICL-670 is covalently attached. Consequent to PEG acrylate grafting, the chelators become non-permeable to cells and should demonstrate a vastly increased vascular retention time. Vascular retention time and kidney clearance is readily manipulated by changing both the molecular weight of the PEG-acrylate backbone and the degree of side chain PEG binding, which effects the Stokes’ radius of the molecule. The PEG side chains are attached via biodegradable bonds so as to allow the slow cleavage and removal of the PEG and poly acrylate via the kidney. (Courtesy of Dr. Scott) (136).

Current low molecular weight metal chelators under development for clinical usage are beset by problems of insolubility in aqueous solutions, such as water and physiological plasma. The novel chelator SK-1, however, does not have such solubility problems, comparing the solubility in water of low molecular weight ICL-670 (3 mg/ml) and SK-1; ICL-670, as monomer is insoluble, however SK-1 is fully soluble in water. In addition, the equivalent ICL-670 content in SK-1 (5 mg/ml) in comparison with the 1mg/ml for ICL-670 reveals vastly improved solubility. The iron binding was not affected by the conjugation of PEG to ICL-670. Consequent to SK-1’s improved solubility characteristics; improved therapeutic dosages can be achieved by the chelation structure of the present invention. (Figure 1.19)
Figure 1.19. Compared solubility of ICL-670 with polyethylene glycol conjugated ICL-670 (SK-1) and iron binding of SK-1. Shown in A is the iron binding of SK-1, B is the compared aqueous solubility of monomer ICL-670 and SK-1. Monomer ICL-670 is insoluble in water; SK-1 is fully soluble with no precipitate. (Modified from Scott et al., US Patent 2007) (136).

As part of the ongoing research by Scott et al, PEG was attached to DFO (P-DFO), and the detailed in vitro evaluation of a novel class of high molecular weight iron chelators based on DFO and PEG was reported (140) (146). Reversible addition fragment chain transfer (RAFT) copolymerization afforded polymer conjugates (P-DFO) with a well-controlled molecular weight (27–127 kDa) and substitution of DFO (5–26 units per chain) along the copolymer. Biocompatibility assays including coagulation profiles and cell viability assay, using HUVEC cells of the various P-DFO samples, were reported. Furthermore, the efficacies of P-DFO iron binding properties were compared to DFO by measuring the spectral properties upon binding to Fe$^{3+}$, and the inhibitory effect of Fe$^{3+}$ driven hemoglobin was also determined. Degradation of the P-DFO conjugates via cleavable ester linkages between the polymer backbone and the PEG side chains was evaluated using gel permeation chromatography (GPC) and NMR. Since the chelating ability of DFO remains intact after
conjugation to the copolymer backbone, these macromolecular, blood compatible, and degradable conjugates are promising candidates for long circulation time, non-toxic iron chelators (140, 146).

1.6.4 Iron Shuttle Chelation Therapy

Over the past few decades, it has been well documented that regular RBC transfusions, with adequate iron chelation therapy, have led to dramatic improvements in quality of life and survival rate of thalassemia major patients. Even though thalassemia major treatment corrects severe anemia, it leads to secondary iron overload with time and damages vital organs, including heart, liver, kidney, and endocrine glands. Although patients complied with the monotherapy of DFO, there is evidence that many die of cardiac complications. The idea that combined therapy of DFO and L1 may improve overall excretion of iron, while not producing a negative overall iron balance was then investigated (147,148). The efficiency of combination therapy is based on the log stability constant of chelator to iron, the different tissue distribution of chelators, and the increase in the total time for which chelators are active. However, apart from the expected additive effect, in vitro and metabolic iron balance studies have suggested the possibility of synergy between the two chelators; a “shuttle effect” leading to increased iron excretion. Access of the two chelators to different iron pools would contribute to both additive and synergistic effects (42, 147).

With the idea of a shuttle effect and combination chelation therapy appearing to be effective in patients, and improved, less toxic, high-molecular weight iron chelators commercially available for research, Scott hypothesized that iron shuttle chelation therapy may be an alternative treatment for thalassemia (42). This hypothesis was based on intraerythocytic iron chelation using a novel iron chelation shuttle system (low affinity cell
permeable), and docking (high affinity cell impermeable) chelators. The chelation of free iron from within the RBC can slow or prevent the injury to thalassemic RBC resulting in improved RBC survival within the circulation system.

This shuttle system employs small cell-permeable iron chelating (shuttle) agents (Table 1.10), which remove free redox available iron and hand it off to very large cell impermeable (i.e., extracellular) docking chelators for eventual clearance via the kidney. (Figure 1.20) Scott further hypothesized that this iron shuttle system will give rise to a synergistic increase in iron removal over that of the individual compounds. Increases in circulation time of the individual’s own RBC would decrease the degree of anemia in a significant number of patients, potentially preventing life-long transfusion therapy. At the tissue level, decrease in iron buildup will reduce iron accumulation-arbitrated toxicity and tissue damage. This reduction will further improve total metabolism and the normal functioning of tissues, enabling a higher quality of life in patients (42).

Table 1.10. Potential shuttle chelating agents. (42, 129, 130)

<table>
<thead>
<tr>
<th>Pharmacological agents</th>
<th>Metal binding log stability constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly-His-Lys (GHK)</td>
<td>ND</td>
</tr>
<tr>
<td>2,3- Dihydroxybenzoic acid (2,3- DHB)</td>
<td>19.9</td>
</tr>
<tr>
<td>Pyridoxal Isonicotinoyl Hydrazones (PIH) and its derivatives</td>
<td>35.7</td>
</tr>
<tr>
<td>2,2’-bipyridyl</td>
<td>17.4 [Fe2+]</td>
</tr>
<tr>
<td>1,2- dimethyl-3-hydroxypyrid-4-one &amp; derivatives (e.g., L1, CP20)</td>
<td>36</td>
</tr>
<tr>
<td>1-hydroxypyridine 2-one</td>
<td>27</td>
</tr>
<tr>
<td>CP502</td>
<td>34.3</td>
</tr>
<tr>
<td>ICL670</td>
<td>38.6</td>
</tr>
<tr>
<td>Dexrazoxane (ADR-925)</td>
<td>18.2</td>
</tr>
<tr>
<td>N-N-bis(2-hydroxybenzyl) ethylenediamine –N-N diaceticacid (HBED)</td>
<td>40.0</td>
</tr>
<tr>
<td>Deferrioxamine (DFO, Desferal®; Deferoxamine)</td>
<td>30.6 (Fe)</td>
</tr>
<tr>
<td>O-Trensox</td>
<td>30.9</td>
</tr>
<tr>
<td>Hemopexin (endogenous compound)</td>
<td>ND</td>
</tr>
<tr>
<td>Chloroquine (CQ) and Chloroquine derivatives</td>
<td>6.51</td>
</tr>
</tbody>
</table>
Figure 1.20. Desferoxamine covalently linked to starch (S–DFO) has an iron binding affinity virtually identical to free DFO (Affinity constant for Fe$^{3+}$ > $10^{-30}$). The current drug has DFO constant of $-40$ mM. The plasma half-life of S–DFO can be engineered to range from several hours to several days. Starch is effectively broken down by serum amylase for clearance of the DFO-bound iron via the kidneys. (Modified from Scott, 2001) (42)

Figure 1.21. Scheme representation of the iron shuttle concept. Intracellular or membrane-associated heme and iron is bound by small RBC-permeable shuttle compounds which exit the RBC via random diffusion. In the extra-cellular space, a large RBC-impermeable, high-affinity chelating agent (e.g., HES-DFO, S-DFO, P-DFO) removes the iron/heme from the weakly bound shuttle compounds for clearance from the body. (Modified from Scott, 2001) (42).
1.7 Summary

As we have discussed in this chapter hemoglobinopathies are an important issue in public health of many developing countries, especially the Maldives. There are millions of thalassemia patients who require safe regular blood transfusions to stay alive. But transfusion alone does not have a fully curative advantage in chronically transfused patients, as they experience iron accumulation in vital organs leading to organ failure and death. To treat these patients, along with regular blood transfusions, iron chelation therapy (ICT) is equally important. Regular blood transfusions and ICT proves to augment survival of the patients as they age. Currently available ICT has very significant, therapeutic benefits, however there are issues of high toxicity, reduced plasma retention time, availability, route of application and high cost of production, encouraging researchers to find an alternative to currently available iron chelators. As we have discussed HMW polymer-based iron chelators may improve the future of iron chelation therapy, while reducing toxicity and increasing plasma residence time, and may prove to have therapeutic advantages compared to currently available chelators. Some of the preliminary research on HMW chelators shows their promising future.

Pharmacological interception of intraerythrocytic iron in β-thalassaemic RBC may substantially prevent or diminish iron-dependent injury to the erythrocyte and its progenitor cells. This may subsequently reduce the necessity for blood transfusions. Elimination of iron-mediated tissue injury will reduce organ failure and death of patients. Furthermore, the development of interventions that improve effective iron chelation while minimizing chelator toxicity may have significant therapeutic effects in countries such as the Maldives.
Chapter 2: Hypothesis and Specific Aims

2.1 Overview

Our hypothesis is based on previous studies, existing literature, knowledge of the treatment of hemoglobinopathies and the significant role of iron in these pathological conditions. We hypothesize that the chelation of misplaced iron, that is iron that has escaped the protective protein envelope (globin) of hemoglobin, from within the RBC can slow or prevent injury to thalassemic RBC, resulting in a higher rate of RBC survival within the circulation and tissues, as well as the protection of immune competence. We also believe that iron chelation therapy can improve the safety of transfusion medicine by chelating redox-available iron. To accomplish these tasks, we propose an improved iron chelation therapy using novel high molecular weight chelators and available low molecular weight chelators. The prolonged circulation time of an individual’s RBC would decrease the degree of anemia in a significant number of patients, potentially negating the need for life-long transfusion therapy. In addition, the enhanced iron chelation therapy can treat secondary iron overload (e.g., in the liver) arising from transfusion therapy. We believe that improved iron chelation therapy will increase tissue metabolism and ameliorate the quality of life of the thalassemic individual. Iron chelation effectively binds and removes free and complexed iron/heme, preventing both redox-driven damage and immunosuppression. We also believe that iron chelation can detoxify blood products, such as platelets, in underdeveloped countries where there are inadequate resources to store and supply safe blood.

In this study, we will investigate the improved chelation therapy of “misplaced” iron using three different models. The efficacy of iron chelators will be assessed using RBC and the hemoglobin model. We will also assess the effect of secondary iron overload by using the
HepG2 (liver cells) tissue culture cell model and the impact of iron on immune modulation on antigen presenting cells through the dendritic cell model.

2.2 Global Hypothesis

Excess and/or misplaced iron mediates the pathophysiology of hemoglobinopathies, such as thalassemias. Improved chelation therapy of this redox active iron within the abnormal red blood cell or systemic iron overload (e.g., liver) that arises as a consequence of chronic transfusion therapy will prevent cellular/tissue damage. Moreover, we hypothesize that this “misplaced iron” results in immune dysfunction. Immune dysfunction arises from both the phagocytosis of damaged RBC and the systemic iron overload consequent to transfusions. The development of improved high molecular weight iron chelators may provide better therapeutic value (in other words, be less toxic and require less frequent administration) in developing nations such as the Maldives.

2.2.1 Experimental Hypothesis

Given that improved iron chelation therapy may have therapeutic advantages to current practices in the area, we hypothesize that improved iron chelators will prevent cellular and tissue injury due to bioavailable iron. (Figure 2.1)
Figure 2.1. Scheme of hypothesis. Thalassemias arise from mutations to hemoglobin that result in destabilized hemoglobin and, potentially, life-threatening anemia due in part to iron-driven redox reactions at RBC level (A), also called “primary iron overload.” While a transfusion corrects the anemia, (B) secondary iron overload can occur in parenchymal tissues and organs. Thus, both the primary and secondary pathologies of thalassemia arise from “misplaced” iron. The iron then drives free radical mediated injury to other hemoglobin molecules, as well as the RBC itself. The removal of oxidatively-damaged RBC in vivo occurs primarily via erythrophagocytosis by the mononuclear phagocytic system (MPS). This clearance mechanism may result in negative immunoregulatory effects (C).
2.3 Specific Aims

Experiments were conducted using three different models to observe the efficacy of iron chelators and the effect of iron chelation on cells. In order to achieve these goals, the following specific aims were determined.

2.3.1 Primary RBC Iron Overload

RBC and Hemoglobin Model

1) The effects of bioavailable iron will be evaluated and the prevention of cell injury by chelators will be examined in this model. (Figure 2.2) The effect of oxidized RBC on antigen presentation will also be monitored to determine:

1) UV Visible Spectra Fe$^{3+}$ chelator binding;

2) Thermogravimetric analysis (TGA) of P-DFO and S-DFO

3) Fe$^{3+}$ mediated hemoglobin oxidation in RBC lysate (hemolysate) and the inhibitory effect of iron chelators;

4) Fe$^{3+}$ GSH mediated hemoglobin oxidation in purified hemoglobin and the inhibitory effect of iron chelators;

5) PMS mediated intact RBC lysis and the additive effect of iron chelators;

6) Fe$^{3+}$ mediated lipid peroxidation and the thiobarbeturic acid reactive substance (TBARS) formation of RBC membrane ghost challenged with cumene hydroperoxide; and,

7) The effect of heme and PMS oxidized RBC on antigen processing and the presentation on peripheral blood mononuclear cell (PBMC) proliferation.
2.3.2 Secondary Iron Overload, Tissue Damage

Tissue Culture Model (HepG2 Cells)

II) The effects of iron loading and **chelation** therapy on cell viability and structure will be evaluated to: (**Figure 2.2**)

1) Determine cellular iron uptake in HepG2 cells;
2) Demonstrate cellular iron by the Prussian blue iron stain;
3) Determine the effect of iron chelation on the total cellular iron by Ferene iron assay;
4) Determine the impact of iron chelation on cellular ferritin;
5) Determine the effect of iron and iron chelation on cellular structures and organelles by transmission electron microscopy; and,
6) Determine the impact of iron and iron chelation on cell metabolic viability.

**Figure 2.2. Hypothesized effect of iron on cells in the tissue culture model.** When the cells reach their survivorship phenomena, there will be consequences as a result of the iron increase. These effects can be demonstrated in mitochondrial function, ultrastructure TEM of cells, and iron deposits in either ferritin or siderosome cells.
2.3.3 Immune Dysfunction
Dendritic Cell (DC) Model, Antigen Presenting Cells

III) In this model, the effects of iron loading of APC on immune function will be evaluated. (Figure 2.3) We will also examine the affect of chelators in the prevention and reversal of injury or dysfunction to determine:

1) The effect of heme and PMS oxidized RBC on antigen processing and the presentation on peripheral blood mononuclear cell (PBMC) proliferation.
2) The effect of ferric ammonium citrate (FAC) on dendritic cells;
3) Antigen presentation and/or PBMC proliferation;
4) The impact of iron on surface markers of DC, CD83, HLADR, CD86, and CD80;
5) The effect of iron on the mitochondrial membrane potential of DC; and,
6) The impact of iron on DC viability.

Figure 2.3. Effect of iron on antigen presenting cells (APC), due to erythrophagocytosis of abnormal erythrocytes. Removal of oxidatively-damaged RBC in vivo occurs primarily via erythrophagocytosis by the mononuclear phagocytic system (MPS), including APC. This clearance mechanism may result in negative immunoregulatory effects, such as decreased antigen presentation, activation, and lymphocyte proliferation.
2.4 The Significance of Improved Iron Chelation Therapy

At the moment, the only practical solution for the treatment of millions of hemoglobinopathy patients across the globe is regular blood transfusions with affordable and efficient iron chelation therapy. Theories are circulating throughout the scientific community regarding alternative ways to treat thalassemia majors, including gene therapy and BMT. The practicalities of these interventions are very complex and such alternatives are not likely to have a significant influence in the near future, largely because current research priorities lie with diseases that affect people in developed countries, such as cancer and cardiovascular disease, and the prevalence of thalassemia is in under-developed areas of the world. It is important to improve iron chelation therapy in order to advance the quality of life for those millions of patients in need.

Improving the existing iron chelators by conjugating them with biocompatible polymers, the desired longer plasma retention time may be achieved, enabling maximum iron chelation. By chelating intraerythrocytic bioavailable iron and extracellular misplaced iron, we may slow or prevent injury to thalassemic RBC, resulting in a higher rate of RBC survival within the circulation. Increases in circulation time of an individual’s RBC would decrease the degree of anemia in a significant number of patients, potentially negating the need for life-long transfusion therapy. At the tissue level, a significant decrease in iron buildup will reduce iron accumulation-arbitrated toxicity and tissue damage.

The removal of thalassemic RBC in vivo occurs primarily via erythrophagocytosis by the mononuclear phagocytic system (MPS), including the spleen, liver and bone marrow macrophages, and APC (149). This clearance mechanism may result in negative immunoregulatory effects, such as an increased risk of bacterial infections in these patients. By chelating misplaced iron in either thalassemic cells or tissues that may have
immunoregulatory effects, we could prevent the risk of infection and save many patients in third-world countries with inadequate resources (150). In addition, we may be able to utilize iron chelators to improve the shelf life of blood products in developing nations. If these approaches can be successfully implemented by enhanced iron chelation therapy, a significant number of patients will no longer require chronic RBC transfusion therapy, thus completely avoiding subsequent secondary iron overload, toxicity, organ failure, and death.
Chapter 3: Materials and Methods

3.1 Overview

In this chapter, the methodologies used to perform the experiments in order to achieve the specific aims outlined in the previous section will be briefly explained. Most of the methods used in this research are well established and previously published by others in this field.

3.2 Common Methods and Materials

3.2.1 Statistical Analysis

All results are expressed as mean ± standard deviation of the mean (SD). A minimum of 3 replicates was performed for all studies, although in some cases (e.g., microscopy and spectral scan) representative data were presented. Statistical analyses were done using SPSS v. 16.0 statistical software (Statistical Products and Services Solutions, SPSS Inc., Chicago, IL, USA). For comparisons of 3 or more mean values, a one-way analysis of variance (ANOVA) was performed followed by a Tukey post-hoc test for pair-wise comparisons of means. For comparisons of 2 mean values, an independent variable student T-test was used. Significance was determined by a two-tailed p value <0.05.

3.2.2 Flow Cytometry

The flow cytometer was calibrated against standardized samples of fluorescent beads provided by the manufacturer (BD FACS Calibur, BD Biosciences, San Jose, CA, USA). Data acquisition and analysis were performed using Cell Quest software (BD Biosciences, San Jose, CA, USA). In total, 20,000 events were collected per sample.
3.2.3 Colorimetric Analysis

A spectrophotometer, Spectronic Helios Alpha system with high energy and a double beam for ultimate stability in the UV region, was used in all of the experiments (Thermo Fisher Scientific Inc., Waltham, MA, USA). VISION 32, software for Spectronic Helios UV-Vis spectrophotometer, was used to test setup, data collection, data manipulation, and results presentation. In experiments where the wavelength is a UV-Visible spectrum, quartz cuvettes were used; otherwise, polystyrene disposable cuvettes were utilized in most cases. (Sarsted Inc, Montreal, Québec, Canada)

A single-mode microplate reader was used in microplate-based assays, including ELISAs, and cell viability assays. (Molecular Devices, Downingtown, PA, USA)

Absorbance detection measured how much light was absorbed by a sample. Absorbance-based detection has been commonly used to assess changes in color or turbidity, allowing for widespread use in ELISAs and cell viability cytotoxicity assays.

3.2.4 Thermogravimetric Analysis (TGA)

Thermogravimetric analysis (TGA) was performed on TGA Q 500 (TA Instruments, Q series™). Samples were heated from 100 °C to 600 °C at rate of 20 °C per minutes. The sample S-DFO in saline was dialyzed against water for 24 hours with cellulose membrane (MWCO 1kDa), after which the product was lyophilized.

3.2.5 Iron Chelators

3.2.5.1 Low Molecular Weight Iron Chelators

Deferoxamine (DFO) mesylate salt, FW 656.8 (Sigma-Aldrich Canada Ltd. Oakville, ON, Canada), Deferiprone mesylate (L1), FW 139 (Apotex Inc., Toronto, ON, Canada) and N,N’ Di (2 Hydroxy benzyl) “Ethylenediamine –N,N diaceteic acid
monohydrochloride” HBED, FW 461.35 Lot No: B9674014 (Strem Chemicals, Newburyport, MA, USA) were purchased. For experiments, a stock solution of DFO, L1, and HBED was prepared in accordance with the protocols written for the particular experiment. For hemoglobin oxidation scans, chelators were prepared in either water or in the buffer solutions used in the experiment; in intact RBCs or cell culture works (HepG2 or DC) chelators were added directly to the culture medium used in the experiment itself. DFO, L1, and HBED were weighed using a Denver Instrument 30 Company XT Series Model 400D balance (Denver, CO, USA). Samples of each stock solution were prepared freshly for every experiment.

3.2.5.2 High Molecular Weight Iron Chelators

Initially a novel PEG-ICL-670 (SK-1) derivative, a HMW chelator synthesized in Kizhakkedathu’s laboratory was tested for its solubility. Compared with a tridentate oral iron chelator ICL-670 which is insoluble in water, SK-1 is fully soluble in water. Also PEG-DFO conjugates (P-DFO), MW (27-127 kDa), were obtained from Kizhakkedathu’s laboratory (140). Time constraints and practical difficulties hindered the synthesis of large quantities of these polymer-based chelators so commercially available HMW chelator DFO derivatives conjugated to a starch backbone (S-DFO) were obtained (Biomedical Frontiers Lot No: 031005 40SD02, 100 mg/ml in 0.9 g% NaCl pH 5-7, MW 26,000 Da) (143).

3.2.5.3 Iron

For hemoglobin oxidation and lipid peroxidation studies, an ammonium iron (III) sulphate dodecahydrate, Sigma Ultra minimum 99% FW 482.19 (NH₄Fe(SO₄)₂·12H₂O) was used. For cell culture experiments, ammonium iron (III) citrate, (FAC) C₆H₈O₇·Fe.H₃N, FW 265.0 was used (Sigma Aldrich, St. Louis, MO, USA). FAC was used for the iron
loading of HepG2 cells and DC, in order to avoid the precipitation and crystallization of iron salt in cells. In addition, it is well known that iron loading of HepG2 cells with FAC gives a reasonable reflection of the magnitude of in vivo liver iron overload in patients (151).

3.3 RBC and the Hemoglobin Model

3.3.1 Packed Red Cell (pRBC) Preparation

Fresh human whole blood in anticoagulant, acid citrate dextrose (ACD), or sodium heparin were obtained in vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) from consenting, informed, and healthy volunteer donors at the Centre for Blood Research, University of British Columbia, Vancouver, BC and processed immediately. Plasma was separated by centrifugation at 2500 g for 5 minutes at room temperature. Red blood cells (RBC) were washed three times with isotonic saline (NaCl 154 mM). The buffy coat, together with part of the upper RBC layer was removed and discarded after each wash. After three washes, the RBC yield was referred to as packed red blood cells (pRBC).

3.3.2 Determination of Hematocrit (Hct)

Well-mixed pRBC was drawn into two (BD Clay Adams™ SurePrep™) hematocrit capillary tubes by capillary action avoiding air bubbles. The tubes were about 3/4 full. Excess blood was removed with a KimWipes™ or gauze. One end of each tube was sealed with a small amount of clay material at a 90° angle, ensuring that the seal had a perfectly flat bottom. The filled and sealed capillary tubes were placed into the hematocrit centrifuge, the sealed ends pointing toward the outside of the centrifuge. Duplicate samples were placed opposite each other in order to balance the centrifuge and the position number of each specimen was recorded. Capillary tubes were centrifuged at 12,000 rpm for 5 minutes at
room temperature, using Clay Adams™ Autocrit Ultra 3 Centrifuge Model 420575.

Hematocrit was measured directly from the tubes using the hematocrit reading scales and the volume of pRBCs needed to prepare 20% hematocrit in HBSS was calculated.

3.3.3 Determination of Hemoglobin Concentration

Hemoglobin (Hb) concentration in grams/litre (g/l) was determined by the cyanmethemoglobin method based on Drabkin's assay with cyanide-ferric cyanide solution, spectrophotometrically at a wavelength 540 nm, by a Thermo Spectronic, Helios Alpha UV – Vis Spectrophotometer. Drabkin’s reagents were purchased from Sigma Aldrich (St. Louis, MO, USA). Hemoglobin concentration in g/l was converted to molar concentration by dividing the molecular mass of human hemoglobin 16,114 as Fe monomer (heme), which is equivalent to mol/l. Hemoglobin solutions were prepared in a 5 mM TRIS buffer at pH 7.4 and were adjusted to a final concentration of approximately 40 μM heme at a final pH of between 7.1–7.4 (152).

\[
[Hb] = \frac{OD_{540} \times (\text{Final volume}/\text{Sample volume})}{6.8} = \text{g%}
\]

3.3.4 Hemoglobin Concentration and Red Blood Cell Lysis

Total hemoglobin and percent RBC lysis were determined using Drabkin's Assay. Briefly, Drabkin's reagent converts hemoglobin to cyanomethemoglobin, which absorbs light at 540 nm. Aliquots of phenozine methosulphate (PMS) and iron+/- chelator treated 20% hematocrit RBC solutions were removed and immediately mixed with Drabkin's reagent to determine the total hemoglobin concentration (153). The remaining cell suspension in the aliquot of each sample was centrifuged, and the supernatant was removed and mixed with Drabkin's. Both total and supernatant hemoglobin concentrations were calculated from the
OD at 540 nm as grams of hemoglobin per 100 ml of solution (g%):

\[ \text{[g\% hemoglobin]} = \text{OD}_{540} \times (\text{Final volume} / \text{Sample volume}). \]

6.8

Subsequently, the supernatant hemoglobin was calculated as a percent of total hemoglobin, in order to give percent RBC lysis:

\[
\text{Percent RBC lysis} = \left( \frac{\text{Supernatant hemoglobin}}{\text{Total hemoglobin}} \right) \times 100
\]

3.3.5 Preparation of Purified Hemoglobin

One ml washed pRBC were taken into a Falcon 5 ml polystyrene round-bottom tube (12 x 75 mm). Packed RBC was lysed by repeated flash freezing and thawing 3 times in a dry ice-acetone bath and in hot water. The 1 ml lysate was brought to 2.5 ml with saline and loaded to PD-10 desalting column previously equilibrated with 25 ml isotonic saline; PD-10 desalting column was obtained from Amersham Bioscience (Piscataway, NJ, USA). Purified hemoglobin was eluted with 3.5 ml isotonic saline. Approximately 2.5 ml of purified hemoglobin yield obtained were aliquoted in 150–250 µl and stored in a freezer at -80 °C. (Figure 3.1)
Figure 3.1. Preparation of packed red cell (pRBC) and purified hemoglobin. RBC were washed three times in saline. Washed pRBC, lysed by flash freezing and thawing, were applied to a pre equilibrated, Sephadex desalting column. Purified hemoglobin fractions were eluted and stored at -80 °C (15) (140).

3.3.6 Fe\(^{3+}\) Mediated Hemoglobin Oxidation and the Inhibitory Effect of Iron Chelators

Iron-driven hemoglobin oxidation provides a high throughput method for measuring the efficacy of both low and high molecular weight chelators. In addition, misplaced iron (trace amounts derived from the autoxidation of hemoglobin) promotes further hemoglobin oxidation and results in destruction of the sickle and thalassemic RBC.

Hemoglobin solutions were prepared from a purified hemoglobin in 5 mM TRIS buffer at pH 7.4 and were adjusted to a final concentration of approximately 40 μM heme at a final pH of between 7.1–7.4. Hemolysates were prepared by hypotonic lysis of pRBC in H\(_2\)O and were adjusted to a final concentration of approximately 40 μM heme at a final pH
of between 7.1–7.4. Unlike hemolysate Fe$^{3+}$ mediated hemoglobin oxidation, purified hemoglobin does not show significant hemoglobin oxidation, and therefore a reducing agent such as glutathione (GSH) is used. Fe$^{3+}$ GSH driven oxidation and Fe$^{3+}$ mediated hemoglobin oxidation in hemolysate was quantified by spectrophotometric analysis (500–700 nm) (15). The scans were done at fixed method selected from the dual beam spectrophotometer, with a cycle time of 31 seconds, for 10 cycles. The test reactions were carried out in disposable polystyrene cuvettes, with an admixture of hemoglobin solution or hemolysate to prechelated chelators, Fe$^{3+}$ and reduced glutathione. The concentration of oxyhemoglobin, methemoglobin, and hemichrome was calculated from the absorption spectra according to the method of Winterbourn (154). *(Figure 3.2)* Different concentrations of chelators were used to examine whether chelation of free iron (ammonium iron(III) sulphate dodecahydrate NH$_4$Fe(SO$_4$)$_2$.12H$_2$O) could inhibit hemoglobin oxidation. The calculations were made using µM extinction coefficients as follows:

- **Oxyhemoglobin (µM)** = 119(OD577-OD 700)-39(OD630-OD700)-89(OD560 -OD700)
- **Methemoglobin (µM)** = 28(OD577-OD 700)-307(OD630-OD700)-55(OD560 - OD700)
- **Hemichrome (µM)** = 133(OD577-OD700)-114(OD630-OD700)-233(OD560 -OD700)
Figure 3.2. Scheme of hemoglobin oxidation spectral scan. Purified hemoglobin solution or hemolysate were scanned by dual beam spectrophotometer, after adding Fe$^{3+}$ and iron chelators to either hemoglobin solution or hemolysate. The spectral changes were recorded to calculate oxyhemoglobin, methemoglobin and hemichrome concentration (15).

3.4 Fe$^{3+}$ Mediated Lipid Peroxidation

3.4.1 RBC Membrane Ghost Preparation

One ml red blood cells were packed and placed in round bottomed, thin-walled Polyallomer centrifuge tubes 25x76 mm (Beckman Coulter, CA, USA) and were lysed with 30 ml hypotonic 5 mM sodium phosphate buffer (Na$_2$HPO$_4$, dibasic and NaH$_2$PO$_4$, monobasic) pH 8.0. The lysed samples were incubated on ice for 10-15 min, then centrifuged at 15000 g at 4 °C for 10 min in a refrigerated centrifuge (Marathon 21K/R, Fisher Scientific, USA) to collect membrane ghosts, after which the supernatant was aspirated. The same cycle was repeated 4-5 times to obtain hemoglobin-free white ghosts. All steps were carried out at 4 °C. Ghosts were stored in aliquots in an -80 °C freezer until analysis (155).

3.4.2 Determination of Thiobarbituric Acid-Reactive Reactive Substances (TBARS)

The thiobarbituric acid (TBA) test measures a secondary product of lipid oxidation, malonaldehyde (MDA). It involves the reaction of malonaldehyde (or malonaldehyde-type
products) with TBA to yield a colored compound that is measured spectrophotometrically. Because the reaction is not specific to malonaldehyde, results sometimes are reported as TBA-reactive substances (TBARS) (156). (Figure 3.3)

![Figure 3.3. Molecular structures of TBARS reactions.](image)

**Figure 3.3. Molecular structures of TBARS reactions.** Lipid peroxidation is usually assayed with thiobarbituric acid (TBA) assay measuring malondialdehyde (MDA) with unsaturated fatty acids in biological samples. The extent of oxidation of different biological samples or organs is related to the component polyunsaturated fatty acids. TBA assays give parallel results for oxidation of unsaturated fatty acids.

One of the consequences of an oxidative stress process is an increase in lipid peroxidation. In this respect, lipid peroxidation is caused by an attack of free radicals upon cell membrane lipids. Measurement of malondialdehyde (MDA), the most abundant product arising from lipid peroxidation, has been extensively used as an index of oxidative stress. We determined lipid peroxidation by thiobarbituric acid-reactive substances (TBARS). Absorbance was determined spectrophotometrically at 532 nm (Thermo Spectronic, Helios Alpha UV –Vis Spectrophotometer, USA). Briefly, the RBC ghost (final protein concentration of 450 μg/ml) +/- Fe³⁺ and iron chelators, challenged with the reducing agent
cumene hydroperoxide (CuOOH) in methanol (MeOH), were incubated for 90 min at 37 °C in a shaking, thermostatic water bath (Thermo Scientific Precision, USA). Samples were taken and mixed with 1 ml TBARS regent previously made with 15 ml trichloroacetic acid (TCA), 0.375 g thiobarbituric acid (4, 6 Dihydroxy 2 Mercapto pyrimidine 98%, Aldrich Chemical Company Inc. Milwaukee WI, USA), 2.15 ml concentrated hydrochloric acid (HCl, 11.9N), and 82.85 ml water. The samples were centrifuged at 1000 g to spin out the TCA precipitated proteins. Clear supernatant was removed to new glass tubes, a marble was placed on the tube top and the mixture was heated to 100 °C for 30 min on a heating block. The optical density was determined at 532 nm, and TBARS were expressed as a percent of positive control and plotted as a bar graph.

3.5 Secondary Iron Overload Cell Culture Model

3.5.1 HepG2 Cell Culture

HepG2 cells, (HB8065 ATCC, Hepatocellular Carcinoma Human, Lot No: 7695685) (ATCC, Manassas, VA, USA) cell line was cultured in T-75 or T-175 tissue culture flasks (Becton Dickenson (BD), Franklin Lakes, NJ, USA) and were grown in a humidified, 5% CO₂ incubator maintained at 37 °C. For cell culture, HepG2 cells were grown in minimal essential media (MEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products, Sacramento, CA, USA), non-essential amino acids, MEM vitamin solution (1X), L-glutamine (2 mM), sodium pyruvate (1X), penicillin (0.292 mg/ml), and streptomycin (100 μg/ml) (Invitrogen). Cells were maintained at a subculture ratio of 1:4 to 1:8 and were passaged every 3-5 days. (Figure 3.4)
General Protocol for Iron Loading HepG2 Cells

**Step 1**  
Grew HepG2 cells in T 75 or T 175 flask in MEM with 10% FBS 1:4 dilution, 70-80% confluence

**Step 2**  
Seed cells in 100 mm Petri dish. 6, 12, 24, 48 or 96 well plates

**Step 3**  
Change the media with Ferric Ammonium Citrate (FAC) 2% FBS

**Step 4**  
Change the media with iron chelators in 2% FBS

**Step 5**  
Trypsinise and harvest the cells, wash 3x with Chelax100 treated PBS

Figure 3.4. General protocol for HepG2 cell culture, iron loading, and iron chelation.  
HepG2 cells cultured in T75 or T175 plates in MEM media with 10% FBS supplemented with sodium pyruvate, essential vitamins, L-glutamine and penicillin and streptomycin, incubated at 37 °C with 5% CO2. After 24 hours cells were rinsed with PBS and treated with FAC and iron chelators as per experimental protocols described in Methods and Materials of particular experiments.

### 3.5.2 HepG2 Cell Culture in Slides and Prussian Blue Iron Staining

HepG2 cells cultured in T175 flasks were trypsinized, and cells were suspended in 10 ml of MEM media with 10% FBS. Cells stained with Trypan Blue were counted using hemocytometer. 1.5x10⁵ cells/ml (50,000 cells/well) were seeded in 8-well culture slides, with 500 μl in each well (Becton Dickinson, Franklin Lakes, NJ). Incubated at 37 °C with 25% CO2, slides were labeled, 2 slides for each time point as T12, T24 and T48. Culture slides were observed under the microscope and found 80% confluent. Medium was aspirated
and the wells were rinsed with 500 µl PBS. HepG2 cells were treated with 0-500 µM of FAC made in medium with 2% FBS in specified wells in the slides as above. After 48 hours of iron treatment, medium was aspirated, rinsed with PBS, and treated with iron chelators.  

(Figure 3.5)

Slides were stained for iron with Prussian Blue iron stain after 12, 24, and 48 hours of iron chelator treatment. Iron stain was performed according to the procedure given by the manufacturer (Sigma Aldrich, St. Louis, MO, USA). Briefly, the medium in the culture slide were drained, and with the apparatus provided by the slide manufacturer, the upper chamber of slides was removed. The slides were immediately fixed with 200 µl 98% ethanol for 5 minutes and then were immersed in deionized water to hydrate the cells. Hydrated slides were overlaid with a working iron stain solution for 10 minutes. Slides were rinsed in deionized water and counter-stained with a working pararosaniline solution for 5 minutes. Slides were rinsed with deionized water, dried, mounted with a cover slip and DPX neutral mounting medium (Sigma Aldrich, St. Louis, MO, USA) for microscopy (157).
Figure 3.5. Scheme of HepG2 cells grown in culture slides and stained with Prussian blue iron stain. HepG2 cells cultured in 8 well chamber specialized culture slides, treated with +/- FAC and iron chelators for the specified time periods. The slides with monolayer of cells were fixed and stained with the iron stain as described in Methods and Materials. DPX mounted slides were analyzed microscopically for iron deposits, representative microscopic pictures were taken.

3.5.3 Determination of Cellular Ferritin

HepG2 cells were iron loaded and treated with iron chelators for 0 and 48 hours, washed with PBS, harvested, and lysed on ice in a lysis buffer containing 150 mM/l NaCl, 50 mM/l Tris, pH 7.4, 1.0% Triton X100, and 1 mM/l PMSF. The cells were incubated in the cold for 15 minutes and sonicated for 30 seconds. The lysates were centrifuged at 7500 g for 10 minutes, and the supernatants were collected and stored at -80 °C until used.
Measurements of cellular ferritin were performed on cell lysates using a human ferritin enzyme-linked immunosorbent assay (ELISA) test kit from BioCheck (Burlingame, CA, USA) (158). The assay system utilizes 1 rabbit antiferritin antibody for solid phase immobilization and a mouse monoclonal antiferritin antibody in the antibody-enzyme (horseradish peroxidase) conjugate solution. Protein concentrations were determined with the Bradford method using the Pierce Coomassie Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). The cellular ferritin is expressed in ng/mg of cellular protein.

3.5.4 Determination of HepG2 Cell Total Iron

HepG2 cell cellular iron (total iron) or Fe$^{3+}$ iron was measured by spectrophotometry using the ferene iron assay. For measurements of total residual iron, the cells were digested with 100 µl of 50% nitric acid at 56 °C for 24 h, following the addition of NaOH to neutralize the nitric acid (159). Sample aliquots were mixed with 40% trichloroacetic acid (TCA) and centrifuged at 12,000 g to precipitate any protein. The supernatants were then removed and mixed with thiourea/ascorbate and Ferene S solutions. The thiourea/ascorbate solution was added to convert Fe$^{3+}$ to the ferrous (Fe$^{2+}$) state; subsequently, Ferene S (3-(2-pyridy1)-5,6-bis (2,5-furylsulfonic acid)-1,2,4-triazine) chelated Fe$^{2+}$ to form a bright blue product, with an absorbance at 594 nm was added. Iron concentrations were calculated using an extinction coefficient of 35.5 mM$^{-1}$cm$^{-1}$ for the Ferene S:iron chelate. The OD 594 readings were converted to iron concentrations with the equation below:

\[
[\text{Fe}^{3+}] = \frac{(\text{OD594} - \text{Blank}) \times 3}{0.0355}
\]
3.5.5 Transmission Electron Microscopy (TEM) for HepG2 Cells Treated With Iron and Iron Chelators

HepG2 cells, grown in a T175 flask were trypsinized and suspended in 10 ml of MEM with 10% FBS. Cells were counted by hemocytometer with trypan blue stain. $2 \times 10^5$ cells/ml (400,000 cells/well) were seeded in 6-well culture plates, with 2 ml in each well. They were incubated at 37 °C with 25% CO$_2$. Culture plates were observed under the microscope and found 80% confluent. After 24 hrs, media aspirated from all the wells was rinsed with 1 ml PBS. HepG2 cells in the plates were treated with 0-500 $\mu$M of FAC made in media with 2% FBS in specified wells in the plate in the same incubation condition. After 48 hours of FAC treatment, the plate was rinsed with 1 ml PBS and then treated with iron chelators prepared in medium and incubated in the same condition for 48 hours. Cells were washed with PBS, and trypsinized, collected into micro centrifuge vials and inactivated with media with 10% FBS. Cell suspension was centrifuged at 3000 rpm to obtain the cell pellet. The cell pellets were fixed with 2.5% gluteraldehyde with one drop of 1 mM CaCl in a 0.2 M sodium cacodylate buffer for 1 hour. The samples were kept in the cold in a shaking mixer (nutating mixer).

After fixation with glutaraldehyde, samples were washed in 0.1 M sodium cacodylate for 10 min x 3 with a new buffer each time. Subsequently, each pellet had the equal volume of a 4% low melting point. Agarose was added for re-suspension at 45 °C, then spun at 12,000 rpm at room temperature for 4 minutes. The agarose-held pellets were then chilled on ice for 0.5 hour and cut into 1-1.5 mm cubes. For post-fixation, samples were incubated in 1% osmium tetroxide in a 0.1 M sodium cacodylate buffer (by adding equal volumes of 2% aqueous osmium and 0.2 M sodium cacodylate buffer, pH 7.2-7.4 just before use) for 1 hour at room temperature. The vials were covered with foil, as the solution was light sensitive.
The samples were then washed in dH₂O for 15 minutes x 3 and dehydrated through increasing concentrations of acetone (30%, 50%, 75% and 100%) for 15 minutes each, and then with 100% acetone for 15 minutes x 3.

Next, each sample was infiltrated with a 1:1 mix of acetone to epon (2.5 ml acetone + 2.5 ml epon) for 1-2 hours and then with a 2:1 ratio mix overnight. The vials were capped with a lid with a hole in the center to allow the gradual evaporation of acetone overnight. Finally, the cells were infiltrated with pure epon for at least five hours or overnight. The infiltrated cells were next placed in embedding boats for polymerization. The blocks were polymerized at 65-70 °C overnight.

Excess epon around the specimen was trimmed away first using a razor blade. Then the block was faced using the EM UC6 ultra microtome (Leica Microsystems, Austria) with glass knives until the surface of the specimen was reached and sufficient area of the specimen occupied the sectioned area. Before thin sectioning, 500 nm thick sections were cut for light microscopy to determine the quality of fixation and if enough cells were collected in each block. Several sections were cut and placed on a glass slide and dried on a hot plate. The dried sections were then stained with Toluidine blue O stain (1.0 g TBO powder + 1.0 g sodium borate + 100 ml dH₂O), rinsed with distilled water, and dried on a hotplate. Sections were then checked under a light microscope.

Blocks with 500 nm thick sections found acceptable under the light microscope then had 60 nm thin sections cut using a Diatome diamond knife (Diatome, Biel, Switzerland). Sections were picked up on copper mesh grids. All sections were lightly stained with 2% uranyl acetate for 2 minutes. Grids were rinsed with distilled water and dried completely. Next, grids were stained for 0.5 minute with Reynold's lead citrate (1.33 g Pb (NO₃)₂ (lead
nitrate) + 1.33 g Pb(NO₃)₂ (lead nitrate) + 30 ml CO₂-free dH₂O) in a small petri dish with NaOH pellets placed inside to absorb CO₂ molecules. Grids were then rinsed with distilled water and dried completely. Stained sections were viewed in a Tecnai 12 TEM (FEI Company, Netherlands) at 80 kV. Digital images were acquired using the Gatan 792 Bioscan model digital camera inserted above the viewing screen. Images were captured in a standardized random fashion. In most cases 5 cells were located for ultra structure and organelles were observed at 5,800x, 59,000x, and 97,000x magnification. All sample-processing procedures in this section were performed inside the fume hood, as toxic reagents are used for the TEM fixation. These reagents were discarded in appropriate waste containers for proper disposal (151)

3.5.6 MTS Cell Viability Assay

Cell viability was determined using an MTS assay kit (CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison WI). After treatments of iron or iron chelators, 20 µl of MTS assay reagent was added to each well and incubated in the same conditions. Absorbance readings were taken after 4 hours of MTS at the wavelength 490 nm by a plate reader. The assay was composed of the tetrazolium compound MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) 2H-tetrazolium) and an electron coupling reagent PMS (phenazine methosulfate). MTS is reduced by viable cells to formazan, measured by the amount of absorbance at 490 nm. Since the production of formazan is proportional to the number of living cells, the intensity of the produced color is a good indication of the viability of the cells (160). (Figure 3.6)
3.6 Antigen Presenting Cells, Dendritic Cell Model

3.6.1 PBMC Extraction and Dendritic Cell Culture

Following informed consent, whole blood was collected from healthy volunteers into 8 ml of vaccutainer tubes containing sodium heparin for peripheral blood mononuclear cell (PBMC) extractions. Peripheral blood mononuclear cells (PBMC) were prepared using Ficoll-Paque Premium (GE Healthcare, Piscataway, NJ, USA) and standard procedure was
followed based on the manufacturing instruction protocol without any modifications. For
dendritic cell preparation, freshly prepared PBMC were plated on a 6-well plate (Becton
Dickinson, Franklin Lakes, NJ), thus allowing the cells to attach to the plate. After 3 hours of
culture, suspension cells were gently washed off the plate and the adherent cells (monocyte
rich cells) grew with IL-4 and GM-CSF (R&D Systems, Minneapolis, MN) in AIM V serum-
free culture medium (Gibco, Invitrogen, Grand Island, NY). IL-4 and GM-CSF were
supplied again onto the cells on days 2 and 5, wherein cells were spun down and responded
in fresh medium supplied with maturation factors including TNF-α, IL-1β, IL-6 (R&D
Systems, Minneapolis, MN) and prostaglandin E₂ (Sigma-Aldrich, St. Louis, MO). On day 6,
stimulators were added on the DC-like cells. During day 7, DC-like cells were harvested and
CD80, CD83, CD86, and HLA-DR expressions were investigated through antibody staining
and flow cytometric assay. (Figure 3.7) (161).

3.6.2 Effect of Erythrophagocytosis and Heme on PBMC Proliferation

Hemin (heme) and PMS oxidized RBC were exposed to PBMC for 2 hours at 37 °C
in order to observe the antigen processing, presentation, and T Cell proliferation. PBMC
(2×10⁵/well) were cultured in 96-well round bottom plates in replicates of six with or without
tetanus toxoid 40 μg/ml at the concentrations indicated. Fifty μl of TT antigen solution were
added to each well, *strep* mutans bacteria were also used as antigens. The plate was
incubated for 72 hours at 37 °C and 5% CO₂. Cells were pulsed, ³H-thymidine was added at
100 μCi/ml, and the cells were harvested 24 hrs later. ³H-thymidine incorporation was
measured by scintillation counting. Results are expressed as counts per minute (CPM).
Figure 3.7. Scheme of PBMC preparation and dendritic cell culture. PBMC were extracted by gradient centrifugation using Ficoll; Paque. PBMC were seeded on six-well plates, and attached PBMC were cultured in GM-CSF (50 ng/mL) and IL-4 (150 ng/mL) for 6 days to become immature dendritic cells. Maturation was induced by adding a Monocyte Conditioning Medium including IL-6, TNF-α, PGE₂, and IL-1 (161).

3.6.3 Effect of Iron and Iron Chelation on Antigen Presentation and T Cell Proliferation

Mature DC-like cells were iron loaded with 200 μM FAC for 24 hours, washed and then treated with iron chelators for another 24 hours. In addition, experiments were carried out with 200 μM FAC pre-complexed with iron chelators and then exposed to DC in order to observe the effect of iron and iron chelation on antigen processing and presentation on antigen-presenting cells. Tetanus toxoid (TT), formaldehyde inactivated tetanus toxin (List
Biological Laboratories Inc, California, USA) were used as antigens for T cell proliferation assay. Antigens TT 3 μg/ml were preincubated with DC-like cells for 1 hour to allow the DC-like cells to capture the antigens before freshly prepared autologous PBMCs were incubated with DC-like cells and antigens.

For staining, PBMC with CFSE (carboxyfluorescein diacetate succinimidyl ester, CellTrace CFSE cell proliferation kit, Invitrogen Molecular Probes, Eugene, OR), and PBMC were re-suspended at 1×10⁷/ml in RPMI 1640 plain medium without serum and other supplements. 1 μl of CFSE at 5 mM stock concentration was used for each 5×10⁶ PBMC. The staining was incubated at 37 °C for 10 minutes. Staining was terminated by adding cold RPMI 1640 culture medium, and the cells were washed twice in RPMI 1640 and re-suspended in an AIM V medium.

PBMCs, DC-like cells incubated with antigens were growing for 14 days and the PBMCs were stained with CD3, and CD4 antibodies (BD Biosciences, San Jose, CA). The cells were analyzed on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA). Data were analyzed using Cell Quest Pro software (BD Biosciences, San Jose, CA) (Figure 3.8) (162).

3.6.4 Effect of Iron and Iron Chelation on DC Mitochondrial Membrane Potential

Mitochondrial injury was assessed by JC-1 staining (Mito Probe™, Invitrogen Molecular Probes, Eugene, OR). This dye, existing as a monomer in a solution emitting a green fluorescence, can assume a dimeric configuration emitting red fluorescence in a reaction driven by the mitochondrial transmembrane potential. Thus, the red fluorescence of JC-1 indicates intact mitochondria, whereas green fluorescence shows monomeric JC-1 that remains unprocessed due to the breakdown of the mitochondrial membrane potential. The
DC exposed to iron and iron chelators were collected and stained by a JC-1 Mito Probe™ assay, as described by the manufacturer. Analysis was performed by FACS scan and mitochondrial function was assessed as JC-1 green (uncoupled mitochondria) or red (intact mitochondria) fluorescence (163).

**Figure 3.8. Scheme of dendritic cells, tetanus toxoid mediated antigen processing and presentation.** DC cultured cells. Treated DC continued with autologous PBMC, a T cell proliferation which therefore potentially causes a helper T cell immune response. Cells are labeled with the fluorescent dye 5,6-carboxyfluorescein diacetate succinimidy ester (CFSE). Those cells that proliferate in response to TT antigen show a reduction in CFSE fluorescence intensity, which is measured directly by flow cytometry. CFSE-labeled autologous donor PBMC is cultured with 3 μg/ml TT in each well for 14 days.
Chapter 4: Results, RBC and Hemoglobin Model

4.1 Overview

The intact RBC and hemoglobin model were used to investigate the efficacy of iron chelators binding iron/heme in an aqueous and lipid environment to inhibit or prevent iron-driven oxidation. The intact, PMS-oxidized RBCs were used to observe the effect of iron shuttle chelation on RBC hemolysis. High throughput screening studies were conducted in vitro via spectrophotometric shifts in the absorption spectra of selected LMW and HMW chelators. Experiments on hemoglobin oxidation were carried out with hemolysates of fresh packed red blood cells (pRBC) and crude purified hemoglobin. Hemoglobin oxidation scans were done with Fe$^{3+}$ and iron chelators. The redox activity of the chelator iron/heme complex was determined via lipid peroxidation. The effects of various compounds of thiobarbituric acid reactive substances (TBARS) formation were examined spectrophotometrically.

4.2 RBC and Hemoglobin Model

4.2.1 Efficacy of Selected Iron Chelators to Bind Iron/Heme in Order to Prevent Iron Mediated Hemoglobin Oxidation in Aqueous and Lipid Environments

While previous studies examined DFO, a hexadentate chelator, little information exists about the relative efficacy of other low molecular weight chelators such as L1 (bidentate), HBED (hexadentate) or high molecular weight DFO-based chelators such as S-DFO and our novel chelator P-DFO.
4.2.2 Iron Binding, UV Spectra of LMW Chelators

As demonstrated in Figure 4.1 panel (A), Deferoxamine (DFO), a hexadentate iron chelator derived from *Streptomyces pilosus* a bacterial siderophore, shows maximal UV spectral absorbance at 429 nm for DFO/Fe\(^{3+}\) complex. Panel (B) N,N'-bis (2-hydroxybenzyl) ethylenediamine-N,N'-diacetic acid (HBED), a synthetic hexadentate iron chelator, shows maximal UV spectral absorbance at 480 nm for HBED/Fe\(^{3+}\) complex. There was a shift upon the addition of 100 μM Fe\(^{3+}\) to 25-100 μM DFO or HBED at pH 7.4 in 5 mM TRIS buffer; however, at 100-300 μM Fe\(^{3+}\)/DFO or Fe\(^{3+}\)/HBED complex, there is no further increase in optical density at λ\(_{\text{max}}\) 429 nm or 480 nm and it reaches a plateau, as shown in panel (D). This observation shows that DFO and HBED are hexadentate iron chelators, which can bind a 1:1 ratio of iron to chelator. HBED has a high iron binding log stability constant of 40.0, compared to DFO’s constant of 30.6. At the 1:1 ratio, DFO and HBED are fully saturated with iron, showing their efficacy to bind iron in an aqueous medium.

Similarly in panel (C), Deferiprone (L1), a bidentate iron chelator, shows a maximal UV spectral absorbance at 461 nm. There was a shift upon the addition of 100 μM Fe\(^{2+}\) to 25-300 μM L1 at pH 7.4 in 5 mM TRIS buffer; however, at 300–400 μM Fe\(^{3+}\)/L1 complex, there is no further increase in optical density at λ\(_{\text{max}}\) 461 nm and it reaches a plateau. This observation shows that L1 is a bidentate iron chelator; at ratios of 1:1 and 1:2 (iron:chelator) the complex remains redox active. However, at a ratio of 1:3, the ligands are fully saturated with iron and become redox-inactive. The log stability constant of L1 is 36.0. Since L1 is a low molecular weight, cell permeable, iron chelator it is important to have a high enough ratio of chelator to iron to prevent iron-driven oxidative damage to cells. Perhaps previously
reported L1 liver toxicity may be due to the lack of enough chelator to fully inactivate iron in the liver.
Figure 4.1. UV–Visible spectra of Fe(III), DFO, HBED and L1 binding in aqueous medium. (A) DFO, (B) HBED, (C) L1, (D) Fe$^{3+}$ chelator complex lambda maxima with absorbance values of 25, 50, 100, 200, 300 and 400 μM [DFO, HBED, L1] - 100 μM [Fe$^{3+}$] binding in a 5 mM TRIS buffer, pH 7.4, respectively.
4.2.3 Iron Binding, UV-Spectra of HMW Chelators

As part of ongoing research to synthesize novel high-molecular weight iron chelators, the efficacy of S-DFO and P-DFO iron binding were also evaluated and compared. The spectral properties of Fe$^{3+}$ complexed P-DFO and S-DFO samples were measured and compared to the single molecule DFO-Fe$^{3+}$ complex. Figure 4.2 shows a comparison of P-DFO, S-DFO and DFO before and after binding to Fe$^{3+}$. A broad absorption peak around 429 nm is indicative that DFO has been successfully bound to Fe$^{3+}$ and occurs in both monomeric and polymer-conjugated DFO. As expected, DFO, P-DFO, and S-DFO samples without Fe$^{3+}$ show no absorption at 429 nm.

![Figure 4.2](image.png)

**Figure 4.2.** UV–Visible spectra of Fe(III) desferrioxamine (DFO), polyethylene glycol conjugate DFO (P-DFO) and starch conjugate DFO (S-DFO). (A) S-DFO, (B) P-DFO, Fe$^{3+}$ chelator complex lambda maxima at 429 nm with absorbance values for [P-DFO, S-DFO] - 250 µM [Fe$^{3+}$] binding in 5 mM TRIS buffer (pH 7.4).

4.2.4 Thermogravimetric Analysis (TGA) of P-DFO and S-DFO

As demonstrated in Figure 4.3, polyethylene glycol conjugated DFO (P-DFO) and starch conjugate DFO (S-DFO) is thermally stable to comparable extent. Samples were heated from 20 °C to 600 °C at the rate of 20 °C per minute. P-DFO degradation started at
190 °C, 50 % weight loss at 365 °C and 100% degradation at 446 °C. Similarly, S-DFO degradation started at 176 °C, 50 % weight loss at 321 °C and complete degradation at 363 °C. Pattern of degradation curves, shows that P-DFO is more stable than S-DFO. This difference in thermal stability is because of different structural units of the polymers.

**Figure 4.3.** P-DFO and S-DFO show thermal stability by TGA analysis. Thermo-Gravimetric Analysis (TGA) thermogram of, P-DFO and S-DFO with a heating rate of 20 °C/min in a nitrogen atmosphere.

4.2.5 Cytotoxicity of DFO, S-DFO and P-DFO

Cell viability was evaluated using HepG2 cells. DFO, P-DFO and S-DFO of 0-650 μM were applied to 100% confluent HepG2 cells for 48 hours and MTS assay was conducted to see the viability and effect of iron chelators on these cells. DFO of <25 μM is toxic and thus reduced the viability of cells to 20%. However, at up to 600 μM P-DFO and S-DFO the cells remained fully viable. Cell viability studies in the presence of either DFO or P-DFO were performed; concentrations of DFO were compared to similar concentrations of DFO covalently bound to the copolymers. HepG2 were left to grow in medium for 48 hours with chelators, resulting in a significant difference between the toxicity profiles of singular DFO vs. P-DFO and S-DFO. The cell viability profiles of DFO, P-DFO and S-DFO are shown in (Figure 4.4). As expected, unbound DFO caused a sharp decrease in cell proliferation at low concentrations. At 30 μM, cell survival is approximately 66%, while hardly any cells survive
beyond 25 µM. However, similar concentrations of DFO conjugated to the copolymer backbone show no discernable toxicity, since cell survival is effectively >95% up to 300 µM of DFO content. At >600 µM of DFO equivalent concentration, the percentage of cell survival is approximately ~90% in both P-DFO and S-DFO. There were no significant differences in the toxicity profiles between the two HMW chelators used in this study.

This data strongly suggests that polyethylene glycol conjugated DFO and starch conjugated DFO both are more protective and less toxic to cells than DFO alone.

4.2.6 PMS-Mediated RBC Lysis: The Affects of Combination Iron Chelation Therapy

The oxidative stress caused by phenazine methosulphate (PMS) on intact RBC is illustrated in this section. PMS causes intracellular Ca\(^{2+}\) to rise and dramatically increases membrane permeability for K\(^{+}\) by activating Gardos channels; this activation results in RBC dehydration, rigidity and lack of elasticity, which leads to osmotic resistance, resulting in hemolysis. Experiments were conducted to see whether this oxidative stress, that led to RBC lysis and was caused by 50 µM PMS treated 20% hematocrit pRBC, can be inhibited by iron...
chelators, both cell permeable (L1 and HBED) and cell impermeable (S-DFO). After 30 hours of incubation with HBSS at 37 °C 50 µM, PMS treated samples showed 3.9% RBC lysis. L1 and HBED did not show an inhibitory effect on RBC lysis when they were treated singly, but 100 µM L1 and HBED combined with 1 mM S-DFO had a significant additive effect of 30-33% on L1 and HBED respectively, decreasing RBC lysis in 50 µM PMS treated cells. (Figure 4.5)

Currently, iron chelation therapy is aimed at reducing iron in tissues and organs, but not at globin α-chain derived iron in erythrocytes and erythroblasts. It has been reported by Scott et al., 1990 and 1993, that α-chain derived iron causes oxidative damage in thalassemic cells. This data suggest that improved, less toxic, cell impermeable iron chelators with a high affinity for iron, combined with cell-permeable iron chelators with a low affinity for iron, could be a valuable therapeutic intervention to treat secondary iron overloaded patients, such as those with thalassemia and sickle cell disease.

Figure 4.5. Additive effects of iron shuttle chelation on lysis of RBC, treated with PMS, DFO, L1 and S-DFO. RBCs were first incubated in HBSS at 37 °C in the absence of 50 µM PMS with 100 µM L1, HBED and 1 mM S-DFO or in the presence of 50 µM PMS + 1 mM S-DFO, alone or combined with 100 µM L1 or HBED at 37 °C, for an additional 30 hours. The values shown are the mean ± SD of 3 independent experiments.
4.2.7 Fe$^{3+}$ Mediated Hemoglobin Oxidation and the Inhibitory Effect of Iron Chelators

In agreement with Scott et al., 1993-1995, it was also demonstrated that Fe$^{3+}$ is a potent accelerator of hemoglobin oxidation in hemolysates because of the well-documented catalytic activity of free iron and the fact that iron chelation can inhibit iron-driven oxidation by loose iron. To determine experimentally whether the iron might interact with the endogenous hemoglobin in an autocatalytic oxidation reaction, RBC hemolysates were challenged with Fe$^{3+}$. As shown in Figure 4.6, the direct addition of small amounts of Fe$^{3+}$ to normal hemolysates (~40 μM heme) caused the rapid oxidation of hemoglobin. The characteristic peak or shoulder at 630 nm on the sequential hemoglobin scans shows accelerated methemoglobin formation with an increase in Fe$^{3+}$ concentration. The time in minutes for the individual scan lines following the addition of Fe$^{3+}$ to the hemolysate are indicated. The photograph taken after 10 minutes of reaction clearly shows the change in color, indicating oxyhemoglobin turning to methemoglobin. (Figure 4.7) No hemoglobin oxidation was observed in the absence of Fe$^{3+}$. The UV spectra shown are of hemolysates made at pH 7.4. Importantly, the iron-mediated oxidation of hemoglobin was completely inhibited by the addition of deferroxamine, a potent iron chelator (15), L1 and HBED.
Figure 4.6. Fe$^{3+}$ was found to be a potent accelerator for hemoglobin oxidation in hemolysates. Shown are the sequential hemoglobin scans (A, B and C) D percent oxyhemoglobin concentration, of hemolysates made of packed red blood cells (pRBC) treated with 100, 175 and 250 µM Fe$^{3+}$. The characteristic peak or shoulder at 630 nm shows accelerated methemoglobin formation with an increase in Fe$^{3+}$ concentration. The time in minutes, for the individual scan lines following the addition of Fe$^{3+}$ to the hemolysate are indicated. No hemoglobin oxidation was observed in the absence of Fe$^{3+}$. The UV spectra shown are of hemolysates made at pH 7.4.
Figure 4.7. Photographic display of Fe^{3+} as potent accelerator for hemoglobin oxidation in hemolysates. Shown are the cuvettes (1, 2, 3, and 4) of hemolysates made of packed red blood cells (pRBC) treated with 100, 175 and 250 μM Fe^{3+}. The photograph is taken 10 minutes after the reaction. The control hemolysate does not change color, which is red; the addition of Fe^{3+}, changed the color to brown with dose response, which shows that the hemoglobin has changed to methemoglobin.

4.2.8 Iron-Mediated Hemoglobin Oxidation in Hemolysate and the Inhibitory Effect of Deferiprone (L1) and HBED

Deferiprone (L1), a bidentate iron chelator, renders iron redox inert at a ratio of 1:3 (iron: chelator). Shown here is the increased hemoglobin oxidation with 250 μM [Fe^{3+}] in hemolysate. (Figure 4.8) This hemoglobin oxidation was inhibited, however, by a ratio of more than 1:3 (Fe^{3+}: L1). L1 between 25-1000 μM shows the dose response of an inhibition of 250 μM Fe^{3+} mediated hemoglobin oxidation. The ratio of chelator to iron in part underlies the erroneous reports regarding L1 toxicity. Similarly as shown in Figure 4.9, the
hexadentate chelator HBED 1:1 ratio has inhibited ~90% of the hemoglobin oxidation normally caused by 250 µM Fe³⁺ in hemolysate. HBED >500 µM has virtually inhibited all the hemoglobin oxidation.

Figure 4.8. Iron mediated hemoglobin oxidation in hemolysate and the inhibitory effect of deferiprone (L1). Fe³⁺ was found to be a potent accelerator for hemoglobin oxidation in hemolysates. Shown is the oxyhemoglobin concentration following the addition of 250 (—) µM [Fe³⁺] in hemolysate made of pRBC. No hemoglobin oxidation was observed in the absence (---) of added Fe³⁺. Similarly, in the presence of deferiprone (L1), an iron chelator 25 (●), 50 (■), 100 (□), 175 (◆), 250 (◇), 500 (▲), 750 (△) and 1000 (▼) µM [L1] inhibited iron mediated hemoglobin oxidation normally induced by 250 µM [Fe³⁺] in a dose response manner. Hemoglobin concentration was adjusted to ~40 µM heme at pH 7.4.
Figure 4.9. Iron mediated hemoglobin oxidation in hemolysate and inhibitory effect of HBED. Shown is the oxyhemoglobin concentration following the addition of 250 (—) μM [Fe$^{3+}$] in hemolysate made of pRBC. No hemoglobin oxidation was observed in the absence (---) of added Fe$^{3+}$. Similarly in the presence of HBED an iron chelator 25 (●), 50 (■), 100 (□), 175 (◆), 250 (◇), 500 (▲), 750 (△) and 1000 (▼) μM [HBED] inhibited iron mediated hemoglobin oxidation normally induced by 250 μM [Fe$^{3+}$] in a dose response manner. Hemoglobin concentration was adjusted to ~40 μM heme at pH 7.4.
4.2.9 Iron-Mediated Hemoglobin Oxidation in Purified Hemoglobin and the Inhibitory Effect of Iron Chelators

To discover whether the “antioxidant” reduced glutathione (GSH) had an inhibitory effect on hemoglobin oxidation, GSH was added to the purified hemoglobin solution. As demonstrated in Figure 4.10, GSH is a potent accelerator of hemoglobin oxidation in a dose-dependent manner. This oxidation was further enhanced by hydrogen peroxide (H₂O₂), a normal byproduct of hemoglobin auto-oxidation.

However, in contrast to iron-only mediated hemoglobin oxidation, as previously reported by Scott et al. in 1995, purified hemoglobin does not show any significant hemoglobin oxidation with 100 μM Fe³⁺ alone. However, the inclusion of 100 μM Fe³⁺ with a reducing agent such as GSH has shown that 0.1-10 mM GSH causes a dramatic decrease in % oxyhemoglobin concentration in a dose-response manner. Within 5.16 minutes 1 mM GSH/Fe³⁺ reduced oxyhemoglobin <40%, while 5-10 mM GSH/Fe³⁺ reached 0% with 3.5-1.5 minutes.

In contrast to low GSH concentration, up to 1 mM GSH alone, there is no hemoglobin oxidation in purified hemoglobin by GSH; on the other hand, 5-10 mM GSH itself displays a dramatic increase in hemoglobin oxidation and a significant reduction of oxyhemoglobin concentration within 5 min. This data suggests that glutathione acts as a pro-oxidant for hemoglobin oxidation. Glutathione enhances free iron hemoglobin oxidation reactions. This is perhaps the self-amplifying oxidation reaction observed in thalassemic RBC due to excess α-globin chain precipitates and released free iron, which can react with the abundant amount of glutathione present in RBC.
Figure 4.10. Fe$^{3+}$ glutathione (GSH) driven hemoglobin oxidation. GSH has been shown as a mediator to accelerate Fe$^{3+}$ mediated hemoglobin oxidation in purified hemoglobin, HbA ($\alpha_2$ $\beta_2$) in a dose dependent manner. Shown is the oxyhemoglobin concentration in HbA control (----), 1 (O), 5 (△), 10 (◆) mM [GSH] alone, and following the addition of exogenous 100 (—) $\mu$M [Fe$^{3+}$] in the presence of 0.1 (■) 0.4 (▼), 0.8 (□), 1(●), 5 (▲) and 10 (◇) mM [GSH]. In contrast to purified HbA control, in the absence of GSH there is no significant hemoglobin oxidation with 100 $\mu$M Fe$^{3+}$, however addition of GSH at concentrations > 0.05 mM resulted in severe hemoglobin oxidation. Interestingly 5-10 mM GSH alone has shown considerable amount of hemoglobin oxidation. Hemoglobin concentration was adjusted to ~ 40 $\mu$M heme. The results shown are the mean ± SD of 3 independent experiments.
4.2.10 Fe³⁺ GSH Driven Hemoglobin Oxidation in Purified Hemoglobin and The Inhibitory Effect of Iron Chelators

As demonstrated here, there is no significant hemoglobin oxidation with 100 μM Fe³⁺ or 1 mM GSH alone. Surprisingly, 100 μM Fe³⁺ with 1 mM GSH combined has shown a <30% decrease in oxyhemoglobin concentration within 5.16 minutes of reaction, which is quite significant compared to the control. (Figure 4.11)

Interestingly, with the hexadentate iron chelator desferrioxamine (DFO) and HBED, or starch conjugated DFO (S-DFO), or polyethylene glycol conjugated DFO (P-DFO), DFO equivalent to 40-200 μM completely inhibited normal Fe³⁺/GSH driven hemoglobin oxidation in a dose response manner. DFO/Fe complex has a fully saturated 1:1 ratio and prevents iron mediated oxidative damage by inactivating iron; otherwise the complex can form reactive oxygen species and hydroxyl radicals that have a detrimental effect on cells and tissues. Similarly L1, the bidentate chelator, also inhibited the Fe³⁺ GSH mediated hemoglobin oxidation consistent with the chelator to iron ratio.
Figure 4.11. Fe\(^{3+}\) GSH was found to be a potent accelerator for hemoglobin oxidation in purified hemoglobin. Shown is the oxyhemoglobin concentration following the addition of 100 μM [Fe\(^{3+}\)], 1 mM [GSH], in hemoglobin solution made of crude purified hemoglobin (α\(_2\) β\(_2\)). No hemoglobin oxidation was observed in the absence of Fe\(^{3+}\) GSH. Similarly, the presence of DFO (A), S-DFO (B), P-DFO (C), L1 (D) and HBED (E) inhibited all iron-mediated hemoglobin oxidation as a dose response.

### 4.2.11 The Long-Term Effect of Fe\(^{3+}\) Mediated Hemoglobin Oxidation Inhibition by Deferoxamine (DFO) and Starch-Conjugated Deferoxamine (S-DFO)

There is no hemoglobin oxidation without added iron, but the inclusion of 175 μM Fe\(^{3+}\) shows a <30% decrease in oxyhemoglobin concentration. This oxidation is prevented by the addition of an admixture of 100 μM iron and the chelators DFO and S-DFO 100-1000 μM in hemolysate in minutes. However, the long-term exposure of DFO or S-DFO iron complex showed further oxidation started by the complex itself. DFO/Fe\(^{3+}\) complex in the
samples reached 0% oxyhemoglobin concentration within 5 hours, while S-DFO/Fe$^{3+}$ complex still remained protective.

**Figure 4.12** shows the % methemoglobin concentration after the prolonged incubation of hemolysate with ferrioxamine Fe$^{3+}$-DFO (**Figure 4.12A**) and Fe$^{3+}$-S-DFO (**Figure 4.12B**). Within 5 hours, all the DFO-treated samples reached %MetHb$_{50}$, while S-DFO was below %MetHb$_{50}$ even after 10 hours and 1 mM S-DFO was below 40% methemoglobin after 25 hours. DFO iron complex is a very stable compound, but it shows very significant hemoglobin oxidation with long-term exposure to hemolysate. However starch conjugated DFO shows a prolonged protective effect compared to monomer DFO. This data suggests the importance of high molecular weight, less toxic, non-permeable chelators that bind iron and remain less toxic for longer time.

**Figure 4.12.** Long-term effects of Fe$^{3+}$ mediated hemoglobin oxidation inhibition by deferoxamine (DFO) and starch-conjugated deferoxamine (S-DFO). Shown is the % methemoglobin concentration after prolonged incubation of hemolysate with ferrioxamine Fe$^{3+}$-DFO (A) and Fe$^{3+}$-S-DFO (B). Within 5 hours, all DFO-treated samples reached %MetHb$_{50}$ while S-DFO was below %MetHb$_{50}$ even after 10 hours and 1 mM S-DFO was below 40% methemoglobin after 25 hours. The values shown are the mean ± SD of 3 independent experiments.
4.2.12 Iron-Mediated Lipid Peroxidation Inhibitory Effect of Iron Chelators

The redox activity of the chelator iron complex was determined via lipid peroxidation. The effects of various compounds of TBARS formation were examined spectrophotometrically. Iron chelators and Fe$^{3+}$ reactions of RBC membrane ghost mixtures were challenged with cumene hydroperoxide (CuOOH) in methanol. As shown in Figure 4.13, L1, a bidentate chelator, is redox reactive with 1:1 Fe$^{3+}$ to L1 ratio, which shows an increased TBARS formation with 100 μM CuOOH. However, there is a 1:3 ratio of 100 μM CuOOH inhibited Fe$^{3+}$, L1 and CuOOH mediated lipid peroxidation. In contrast, L1 and CuOOH accelerated Fe$^{3+}$ mediated TBARS formation. The hexadentate chelators DFO and HBED also inhibited iron-mediated lipid peroxidation in a dose response manner. The experiment was carried out in the presence of a normal RBC membrane ghost final protein concentration of 450 μg/ml.

Hexadentate chelator DFO requires >100 μM concentration to significantly inhibit damage arising from 100 μM Fe$^{3+}$-CuOOH. This may be due to trace iron present in the lipid extract as well as partitioning (water: lipid) of the DFO. (Figure 4.14) Similarly water-soluble PEG conjugated DFO (P-DFO) >100 μM significantly inhibited Fe$^{3+}$ mediated lipid peroxidation. (Figure 4.15)
Figure 4.13. L1 effectively inhibited iron mediated lipid peroxidation. Bidentate chelator L1 remains redox active with 1:1, Fe$^{3+}$ to L1 ratio, which inhibits TBARS formation with 100 μM Fe$^{3+}$ and 100 μM [CuOOH] incubated for 90 min at 37 °C. The experiment in lipid-rich environments was carried out in the presence of normal RBC membranes, with a final protein concentration of 450 μg/ml. The values shown are the mean ± SD of 3 independent experiments. * p<0.05, ANOVA compared to + control.

Figure 4.14. DFO effectively inhibited iron mediated lipid peroxidation. Shown is dose response of DFO, Fe$^{3+}$ and CuOOH challenged to RBC ghost. DFO remain redox inactive with a ratio of 1:1 of Fe$^{3+}$ to DFO, inhibiting TBARS formation. TBARS assay done as described in Methods and Materials. The values shown are the mean±SD of 3 independent experiments. * p<0.05, ANOVA compared to + control.
Figure 4.15. DFO and P-DFO compared, efficiently inhibited iron mediated lipid peroxidation. Shown is dose response of DFO, P-DFO, Fe$^{3+}$ and CuOOH challenged RBC ghost. TBARS assay done as described in Methods and Materials The values shown are the mean ± SD of 3 independent experiments. * p<0.05, ANOVA compared to + control.

4.3 Summary

In conclusion, DFO, L1 and HBED chelated free and complexed iron/heme in an aqueous environment. ICL-670 shows extraordinarily poor water solubility as a shuttle chelator. In contrast, HMW SK-1 an ICL-670 derivative used as a HMW chelator shows significantly improved solubility in water as shown in Figure 1.19. Previous work shows that a high-molecular-weight iron chelator i.e., by chemically coupling DFO to hydroxyethyl starch (HES-DFO), vastly extended in vivo circulation and reduced DFO toxicity by several logs, and that the shuttle system works on model β-thalassemic RBC. We have shown that P-DFO and S-DFO similarly chelated free and complexed iron/heme in an aqueous environment. Compared to DFO alone, P-DFO or S-DFO shows ~95% cell viability and reduced toxicity. Thermal stability demonstrated by TGA indicates that both P-DFO and S-DFO have a similar pattern of thermal degradation.

Pharmacological interception of intraerythrocytic iron in β-thalassemic RBC may substantially prevent or diminish iron-dependent injury to the erythrocyte and its progenitor.
cells. This may subsequently reduce the necessity of blood transfusions. Elimination of iron-mediated tissue injury (secondary iron overload) will reduce organ failure and death of patients. As a result, the development of interventions that improve effective iron chelation while minimizing chelator toxicity may have significant therapeutic utility.
Chapter 5: Results, HepG2 Cell Model

5.1 Overview

In this chapter, we assess the effect of secondary iron overload by using the HepG2 (liver cells) tissue culture cell model. Iron uptake and iron loading were demonstrated by applying Prussian blue iron stain to cells grown in tissue culture slides. Quantitative assessment of iron accumulation was achieved by cellular ferritin and ferrozine chelatable iron in acid digested cell lysate. Oxidative damage was assessed by TBARS assay and estimation of total protein and cell viability was measured by MTS assay. The effect of iron overload on the iron chelation of HepG2 cells were also assessed by treating with iron chelators, DFO, L1 and S-DFO alone and in combination for 12-48 hours. Prussian blue iron staining, cellular ferritin, total iron, cell viability, and transmission electron microscopy were done in order to see the organelles and ultrastructural changes due to iron and whether iron chelators can reverse the adverse effects of iron on treated cells.

5.2 Iron Loading in HepG2 Cells

The liver is one of the main organs for storage of excess iron. Iron may be stored in cells as a soluble compound, ferritin or in an insoluble form called hemosiderin. In the case of transfusion iron overload, such as in thalassemia and sickle cell anemia, liver biopsies show hemosiderosis. On the Prussian blue iron stain, ferritin appears as a faint bluish tinge while hemosiderin appears as coarse blue granules. In order to validate our model for secondary iron overload, cultured HepG2 cells (human hepatocellular carcinoma cells) were cultured in tissue culture slides, with or without different concentrations of FAC added to culture medium, to demonstrate iron uptake and accumulation. As shown in Figure 5.1,
the Prussian blue iron stain shows an accumulation of dark coarse blue granules of hemosiderin within HepG2 cells in a dose response manner, treated with 0-1000 μM FAC.

The accumulation of iron in Figure 5.1 resembles that which occurs in secondary hemosiderosis, in which there is an underlying cause for iron accumulation such as hemolysis and transfusions. The iron stain helps to define the pattern of iron deposition and provides a clue to the possible underlying causes of excess iron. These data further help to understand the response to iron chelators and the qualitative changes in iron level in HepG2 cells treated with chelators; iron deposition has been thought to affect response to therapy. The iron stain also gives an estimate of the degree of the iron deposition and the extent of deposition in the cells.
Figure 5.1. Demonstration of iron uptake in HepG2 cells by Prussian Blue iron stain. Shown are a dose response of A, Control, B, C, D, E, and F treated with 100, 200, 400, 500 and 1000 μM FAC. The microscopic images are a Prussian blue iron stain of HepG2 cells grown in culture slides, stained after 48 hours of FAC treatment. The dark blue areas indicated by the arrows are the accumulation of iron in the cells. Images were taken at 200x by a light microscope.
Ferritin is a protein that stores iron and it is a good indicator to assess the amount of iron in cells. As illustrated in Figure 5.2, FAC treatment for 0-72 hours resulted in a dramatic induction of ferritin. Surprisingly, the FAC treatment did not show a dose response effect, as 50 and 400 µM FAC yielded virtually identical increases in absolute ferritin levels at all time points. Moreover, no significant differences were noted in ferritin levels at 24 or 48 hours at concentrations <400 µM FAC. A significant difference was noted between 24 and 72 hours of exposure for 50, 100, and 400 µM FAC with the 72-hour time point showing increased ferritin levels. After 48 hours, 200-300 µM FAC is significant compared to 50 µM FAC (p<0.013-0.03). After 72 hours, 400 µM FAC compared to 50 µM FAC reveals a significant difference (p<0.028). Ferritin does not represent the absolute amount of iron in the cells; however, each ferritin molecule binds ~4500 atoms of iron.

![Figure 5.2. Increased expression of cellular ferritin in HepG2 cells in response to iron accumulation.](image)

HepG2 cells treated with 0-400 µM FAC for 24-72 hours, does not show dose response, but shows absolute identical increase in ferritin at all time points. Ferritin is measured, by ELISA as described in Materials and Methods. The values shown are the mean±SD of 3 independent experiments. * p<0.05, **p<0.01, ANOVA compared to negative control cells.

An increase in total iron levels in cells enhances the risk of cells being oxidatively damaged; this increased risk can be correlated between cellular iron levels and lipid peroxidation. The early significance of iron-mediated cellular damage can be demonstrated
by lipid peroxidation; as free radicals damage the cellular membrane due to oxidation, free iron lipid peroxidation will increase. As shown in Figure 5.3, 0-400 μM FAC treatment for 48 hours resulted in a dramatic induction of TBARS due to lipid peroxidation. Concentrations of >400 μM FAC reveal a significant (p<0.001) difference compared to untreated cells. This data suggests that low concentrations of iron treatment in HepG2 cells have less effect while maintaining cellular function and can accumulate iron.

![Figure 5.3. Iron (FAC) challenge of HepG2 cells resulted in lipid peroxidation and TBARS formation.](image)

The metabolic functions and the cell viability of 0-3 mM FAC-treated HepG2 cells dose response was measured by means of the MTS assay after culturing in the presence of FAC added to medium for 48 hours. As is evident from Figure 5.4, in general the cytotoxic effect on the cell increases with greater concentrations of iron. In comparison, the cytotoxic effects of FAC >2.5 mM were much stronger (p<0.00) with a significant decrease in cell viability.
The iron accumulation by HepG2 cells treated with 200 μM FAC (iron) is time-dependent and increased 2-4 fold after 48-96 hours compared to untreated control cells, indicating a total iron uptake of ~64.4%. (Figure 5.5). To examine the relationship between HepG2 cell iron loading, cellular ferritin, and total cellular protein (Figure 5.5C), cells were incubated in medium supplemented with 200 μM FAC. Under these conditions, there is a time-dependent rise in total cellular iron. (Figure 5.5A) After 48-72 hours, total cellular iron content significantly increases (p≤0.00) in proportion to extracellular iron concentration in the medium after 96 hours (p<0.02). After 48 hours, however, there was an increase in cellular ferritin, but it remained constant between 48-96 hours as a plateau (760.77±63.19 ng ferritin/mg protein and 730.47±33 ng ferritin/mg protein) (p<0.00) respectively. (Figure 5.5B) Surprisingly, there is no significant effect on cellular total protein at any time point compared to untreated control cells and 200 μM FAC treated cells.

The iron loading achieved is significant because it demonstrates the same order of magnitude as that found in liver tissue from Fe-overloaded patients, indicating that the loading of HepG2 cells is a reasonable reflection of the magnitude of in vivo hepatic Fe loading. These data were comparable to that of secondary iron overload patients, such as...
those who suffer from thalassemia. Since 200 μM FAC can achieve significant iron overload while maintaining the metabolic viability and function of cells, 200-500 μM FAC concentration was used for further experiments to observe the effects of combination and single chelator iron chelation therapy.

Figure 5.5. Iron uptake of HepG2 cells treated with FAC, demonstrated by cellular total iron, ferritin and effect on total protein. A is ferrozine chelatable total iron measured by Ferene iron assay, B is cellular ferritin, and C is total protein measured by Coomasie blue protein assay on HepG2 cells treated for 48, 72, and 96 hours with FAC. The data shown represents 3 independent experiments±SEM. *p<0.05 significant by ANOVA compared with untreated cells.
5.3 Iron Shuttle Chelation Therapy on Iron Loaded HepG2 Cells

Based on the data in Figure 5.5, further experiments were carried out using 200 μM FAC as a baseline to iron load cells for 48 hours in order to see the effect of the treatment of iron chelation. As illustrated in Figure 5.6, there are qualitative changes in iron levels in samples treated with 50 μM L1 in contrast to S-DFO, due to the fact that its low cellular permeability has little impact after 12 hours of treatment. However, 50 μM L1+200 μM S-DFO at 12 hours does not seem to have a reduced iron burden relative to 200 μM FAC treated samples. Surprisingly, at 24 hours, combination therapy of 50 μM L1 and 200 μM S-DFO has a significantly reduced iron stain that is near to normal iron load compared to positive control.

In other quantitative studies, significant additive effects of L1 and S-DFO combination chelators in decreasing cellular ferritin for 12-48 hours were observed. (Figure 5.7) At 12 hours there was a 40% decrease, while at 24-48 hours the decrease was 58.29-59.0% (p<00, 0.01) and there was also a synergistic effect of a ~30% reduction after 12 hours of treatment in total iron levels and in iron-treated cells. (Figure 5.8) In vivo iron excretion by these chelators was shown to be effective. Another reason found was that ferrozine assay also measures part of the iron released from L1 and S-DFO iron complexes under acidic assay conditions. Therefore, the iron levels measured by ferrozine reflect the total iron levels in the HepG2 cells, including iron released from transferrin, bioavailable iron if present, and iron released from L1 or S-DFO-iron complexes. As shown in Figure 5.9 the metabolic viability of the cells was not affected by this treatment.
Figure 5.6. Microscopic demonstration of “iron shuttle chelation” in iron-loaded HepG2 cells. Shown is a Prussian blue iron staining of HepG2 cells incubated for 48 hours either alone (Control A, F and K) or with ferric ammonium citrate (FAC) 200 μM (B to O) added to culture media. After 48 hours, they were treated with 50 μM L1 (C, H, M), 200 μM S-DFO (D, I, N) and 200 μM S-DFO with 50 μM L1 (E, J, O) for 12 (A-E), 24 (F-J) and 48 (K-O) hours. The Prussian blue iron stain arrow demonstrates the blue granules of hemosiderin in HepG2 cells. With iron shuttle chelation after 24 or 48 hours, no significant amounts of visible iron were observed. Images were taken at 200x by a light microscope.
Figure 5.7. Additive effect of iron shuttle chelation therapy on cellular ferritin in iron-loaded HepG2 cells. HepG2 cells treated with 200 μM FAC for 48 hours. Treated with 50 μM L1 or 200 μM S-DFO, either alone or combination. The cells were harvested after 12, 24, and 48 hours. Total protein and ferritin measured as described in Methods and Materials. The values shown are the mean ± SD of 3 independent experiments * p<0.05, ANOVA compared to positive control cells.

Figure 5.8. Synergistic effect of iron shuttle chelation therapy on cellular ferrozine chelatable iron in iron-loaded HepG2 cells. HepG2 cells treated with 200 μM FAC for 48 hours. Treated with 50 μM L1 or 200 μM S-DFO, either alone or combination. The cells were harvested after 12, 24, and 48 hours. Total iron measured as described in Methods and Materials. The values shown are the mean ± SD of 3 independent experiments * p<0.05, ANOVA compared to positive control cells.
5.9 Effect of Iron and Iron Chelation on HepG2 Cell Organelle and Ultrastructures

The transmission electron microscopic study of representative samples from FAC-loaded HepG2 cells showed electron-dense material, which is consistent with iron-overload in the cytoplasm and various cell constituents (e.g., lysosomes, mitochondria and cytoplasm). TEM studies observed the effect of iron on HepG2 cell organelles and ultra structures, treated with 0-500 μM FAC, 50 μM DFO, 200 μM S-DFO alone or in combination with DFO, and S-DFO on post FAC treated cells for 48 hours. Clear evidence to demonstrate that in 200-500 μM FAC-treated cells, mitochondrial swelling and cristae widening (Figure 5.10B) in addition to an increase in electron dense materials in lysosomes (Figure 5.11B), results when compared to control cells in our experimental model of iron-overload. In addition, we found that the electron dense siderosomes observed in mitochondria, lysosomes, cytoplasm, and nucleus were significantly reversed by DFO and S-DFO combination therapy. (Figure 5.10E, 5.11E, and 5.12E)

There is a close correspondence between lysosomal iron statuses, the susceptibility of
these organelles to oxidant-induced rupture, and the overall sensitivity of cells to oxidative damage. In contrast, the cytosolic labile iron pool may represent only a small fraction of the total cellular redox-active iron, and is in rapid transit to sites of synthesis of metalloproteins (164). Some observations (Table 5.1) also support the idea that an important, perhaps predominant, intracellular pool of labile, redox active iron is within the lysosomes and mitochondria that has been chelated by DFO. If so, these results imply that the selective chelation or removal of intralysosomal iron may be an effective strategy to protect cells against oxidant challenges (165).
Figure 5.10. FAC challenge results in significant iron uptake within the HepG2 mitochondria that is partially reversed by iron chelators. Shown is (TEM) transmission electron microscopy of HepG2 cells; A, control; B, 500 μM FAC treated for 48 hours; C, 50 μM DFO; D, 200 μM S-DFO; E, 50 μM DFO and 200 μM S-DFO treated for 48 hours; post iron loaded cells. The white box shows M, mitochondria and red box insert shows endoplasmic reticulum ER and ribosome Ri; the arrow b indicates electron dense iron deposits, probably siderosome particles, original magnification 97,000x.
Figure 5.11. FAC challenge results in significant iron uptake within the HepG2 lysosomes that is partially reversed by iron chelators. Shown is (TEM) transmission electron microscopy of HepG2 cells; A, control; B, 500μM FAC treated for 48hrs; C, 50μM DFO; D, 200μM S-DFO; E, 50μM DFO and 200μM S-DFO treated for 48 hours; post iron loaded cells. In the white box, L is lysosome, the arrow indicates electron dense iron deposits, probably siderosome particles, original magnification 97,000x.
Figure 5.12. FAC challenge results in significant iron uptake within the HepG2 nuclear membrane that is partially reversed by iron chelators. Shown is (TEM) transmission electron microcopy of HepG2 cells; A, control; B, 500 μM FAC treated for 48hrs; C, 50 μM DFO; D, 200 μM S-DFO; E, 50 μM DFO and 200 μM S-DFO treated for 48 hours; post iron-loaded cells. The arrow “a” indicates nuclear membrane and “b” electron dense iron deposits, probably siderosome particles, original magnification 97,000x.
Table 5.1. Iron shuttle chelation therapy in HepG2 cells, TEM summary findings

<table>
<thead>
<tr>
<th>Samples</th>
<th>Iron Scale</th>
<th>Observations of Iron</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2</td>
<td>If Control is &quot;2&quot;, adding 400 µM S-DFO brings it to 0; the other combinations bring it to 1</td>
</tr>
<tr>
<td>50 µM Control</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>200 µM S-DFO Control</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>400 µM S-DFO Control</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>500 µM [FAC]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>5</td>
<td>Lysosomes are loaded; Mitochondria has considerable FAC</td>
</tr>
<tr>
<td>50 µM DFO</td>
<td>5</td>
<td>Adding 50 µM DFO gives no response in bringing down FAC; Nucleus is loaded</td>
</tr>
<tr>
<td>200 µM S-DFO</td>
<td>1.5 to 2</td>
<td>Adding 200 µM S-DFO, chelating has some improvement from &quot;5&quot;</td>
</tr>
<tr>
<td>200 µM S-DFO+50 µM DFO</td>
<td>3 to 4</td>
<td>With 200 µM S-DFO, adding 50 µM DFO does NOT improve chelation to &quot;3&quot;</td>
</tr>
<tr>
<td>400 µM S-DFO+50 µM DFO</td>
<td>2</td>
<td>Doubling S-DFO to 400 µM improves chelation.</td>
</tr>
</tbody>
</table>

Iron Scale, 0 = almost NO iron; 5=high iron (as in 500 µM FAC sample)
5.5 **Summary**

In our *in vitro* study of the secondary iron overload model, we have demonstrated that HepG2 cells can be successfully overloaded with <2 mM iron concentration, with minimal effect on cellular function and viability. However >2 mM FAC is detrimental to cell survival. HepG2 cells were iron overloaded with 200-500 μM FAC treated for 48 hours, and treated with iron chelators DFO, L1 and S-DFO, alone or in combination. In this study we have demonstrated the additive effect of iron chelation on cellular ferritin decrease after treatment with 50 μM L1+200 μM S-DFO combination therapy, and also a synergistic decrease in total iron on iron loaded HepG2 cells after treatment was observed. A very significant qualitative decrease in visible iron after iron shuttle chelation treatment was confirmed microscopically from Prussian blue iron stained culture slides. Ultrastructural organellular changes were observed on iron loaded HepG2 cells and combination chelation therapy partially or fully reversed the effects of iron observed in iron loaded cells. The cellular metabolic activities of the cells were not affected during the treatment as demonstrated by metabolic viability assay.
Chapter 6: Results, PBMC and Dendritic Cell Model

6.1 Overview

In this chapter, the immunological effects of “misplaced” iron on antigen processing and presentation are assessed by using iron overloaded/damaged RBC and a dendritic cell (DC) and PBMC model. The effect of iron on immune recognition in antigen presenting cells (DC) was assessed using antigens such as tetanus toxoid (TT). Antigen mediated T cell proliferation was done via $^3$H-thymidine incorporation or flow cytometry using intracellularly (CSFE) stained PBMCs. The effects of iron on antigen processing and presentation were further assessed using control and iron-loaded DC-like cells. Moreover, to assess the effect of iron on the functional aspect of antigen presenting cells, changes in mitochondrial membrane potential were monitored in DC by determining the shift in fluorescence emission and the intensity of the dye JC-1 by flow cytometer. In addition, the metabolic viability of the DC-like cells was assessed by MTS assay.

6.2 Antigen Processing/Presentation is Inhibited Following Erythrophagocytosis of Oxidized RBC

A consequence of unstable hemoglobin in RBC is the production of oxidized hemoglobin and the release of free iron within the damaged RBC. These oxidatively damaged cells are subsequently phagocytized. To determine the functional consequences of iron on the MPS, the effects of heme and oxidized RBC on antigen presentation/proliferation by PBMC was examined. Antigens examined included a low molecular weight antigen (tetanus toxoid; TT) and the very large formalin-fixed Streptococcus mutans (SM) bacterium which requires significant antigen processing for induction of PBMC proliferation. PBMC proliferation was determined by $^3$H-thymidine incorporation.
Importantly, as shown in Figures 6.1 and 6.2, both purified heme and oxidized RBC significantly inhibited antigen mediated PBMC proliferation. For example, 100 μM hemin resulted in a >98% reduction in PBMC proliferation in response to the TT or SM antigen (Figure 6.1A and B). Moreover, 50 μM hemin resulted in ~39% and ~71% reductions in PBMC proliferation for TT and SM, respectively. The effect of 50 μM hemin on SM was greater than that observed with TT, perhaps due to the enhanced iron-mediated inhibition of the more complex antigen processing required for SM. Importantly, these levels of free heme or iron are biologically relevant in the RBC, which has an intracellular iron concentration of ~20 mM. Indeed, 50 μM iron would represent only about a 0.25% degradation of the intracellular globin molecules. Similarly, as shown in Figure 6.2, phagocytosis of oxidized RBC virtually abolished the ability of antigen-presenting cells within the PBMC population to present either the TT or SM antigen. Treatment of normal RBC with an oxidant such as PMS resulted in the oxidation of approximately 30% of the intracellular hemoglobin after 2 hours. Consequent to this oxidation, the RBCs are recognized by phagocytic cells and engulfed. Challenging these phagocytic cells with either TT or SM demonstrated that oxidized, but not normal, RBC’s inhibited antigen processing and presentation as shown by decreased cell proliferation. Indeed, subsequent to phagocytosis of oxidized RBC, PBMC proliferation to TT and SM was almost completely inhibited.
Figure 6.1. Antigen processing/presentation is inhibited by addition of heme. Heme pretreatment of PBMC (2 hours at 37 °C) dramatically inhibits the proliferative response to tetanus toxoid and Strep mutans. The efficacy of antigen processing and/or presentation was assessed by ³H-thymidine incorporation in proliferating T cells. Results shown are a representative experiment of quadruplicate samples.

Figure 6.2. Antigen processing and/or presentation is inhibited following the erythrophagocytosis of oxidized RBC. The efficacy of antigen processing and/or presentation was assessed by ³H-thymidine incorporation. Control and oxidized (50 μM PMS for 2 hours at 37 °C in HBSS) RBC were incubated with autologous serum for 30 minutes at 37 °C and overlayed on autologous PBMC as described in Methods and Materials. Results shown are a representative experiment of quadruplicate samples.
6.3 Derivation of Dendritic Cells (DC) and Characterization

Dendritic cells are the most potent antigen-presenting cells in the immune system and are critically involved in the initiation of primary immune responses, autoimmune diseases, graft rejection, human immunodeficiency virus infection, and the generation of T cell-dependent antibodies. (Steinman, 1991) Human DCs were derived from the adherent fraction of healthy donor PBMC by culture in Aim-V medium containing human granulocyte-monocyte colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4). After 7 days culture, the cell population routinely contained 50 to 90% DC by immunophenotypic and morphologic criteria. As shown in Figure 6.3A, mature DCs show >82% expression of CD80, CD83, and CD86, confirming that these DCs proper immunophenotypic features that are commonly known in mature DCs. We also observed that, as shown in Figure 6.3B and C, the morphology changes as the cells mature; for example, in Figure 6.3B, day 1 the resting cells appear round and small, typical morphology of fresh human PBMC, while in Figure 6.3C at day 7, the cells were exposed to cytokines and they were mature, exhibiting morphologic changes with distinct features, such as irregular shapes with branches of dendrites. Hence these DC-like cells can be used to see the effect of iron on antigen processing and presentation in response to T cell stimulation and proliferation.
Figure 6.3. Culturing of adherent PBMC-derived cells yielded a mature DC-like population as demonstrated by phenotypic expression of CD80, CD83 and CD86. A are the flow profiles (CD80, CD83 and CD86) of the cultured DC-like cells (shaded curve: isotype control; open curve: specific staining for CD markers). The majority of dendritic cells (82%) express high levels of mature dendritic cell markers including CD80, CD83, CD86, and HLA-DR (not shown). The photomicrographs (100x) in B and C show cell morphology at Days 1 and 7 of the maturation culture. DC preparation was done as described in the Materials and Methods.
6.4 Tetanus Toxoid (TT) Mediated Antigen Processing and Presentation in PBMC

The presentation of antigen to CD4$^+$ T cells is most frequently associated with professional antigen-presenting cells (APC) that constitutively express MHC class II such as B cells, macrophages, and dendritic cells. However, several other cell types have been shown to present antigen to CD4$^+$ T cells when MHC class II expression is induced. Tetanus toxoid (TT) is a soluble protein antigen that requires processing before presentation by DC. This processing occurs despite the fact that T cells from these individuals exhibited a potent response to TT in the presence of professional APC. In order to find the optimal concentration of TT, we carried out dose response PBMC proliferation assay, with TT antigen 0.3-10 $\mu$g/ml. As shown in Figure 6.4 control cells without TT showed 1.5% T cell proliferation, while 0.3 $\mu$g TT gave 18.3% and 10 $\mu$g TT gave 48.9%, a 2.6 fold increase in T cell proliferation compared to 0.3 $\mu$g. In additional experiments, we chose 3 $\mu$g TT showing 31.1% T cell proliferation, as it is quite significant compared to the control, and also this amount can reduce the TT reagents needed in other experiments. TT was chosen as antigen in our protocols to carry out further experiments, because TT is an antigen to which most of the people are commonly immunized, especially in North America and a significant immune response can be seen most of the donors.
Figure 6.4. Tetanus toxoid (TT) challenge resulted in a dose dependent increase in PBMC proliferation. Shown is T helper cell (CD3+ and CD4+ double positive) proliferation in response to 0.3 to 10 µg TT. Based on these findings, subsequent experiments utilized 3 µg for TT. Shown are representative flow histograms. Cell proliferation measured by CFSE dilution following 12 days challenge with TT.

6.5 Immune Recognition and T Cell Proliferation in Response to Tetanus Toxoid Antigen

In order for DCs to act as APCs, they must be able to internalize and process antigen. To demonstrate this process in vitro experimentally, we exposed DCs to TT antigen and looked for antigen processing and the presentation of DC’s immune response to TT antigen. T cell stimulation and proliferation is a critical step to establish the immune response to TT antigen. Cell proliferation was measured by CFSE incorporation into DC and PBMC. To examine antigen-specific T cell responses in vitro, DC+TT and PBMC were cultured. After 14 days of incubation, T cell proliferative responses were measured by flow cytometer with CD4+ and CD3+ double positive antibody staining. As shown in Figure 6.5A and B, without
TT antigen there is no T cell proliferation, while TT antigen-exposed cells show a very significant immune response, T cell proliferation of 20.04% respectively. In Figure 6.5C, the resting PBMC lymphocytes remain small and rounded, a morphological feature characteristic of resting, nonproliferative lymphocytes. Also shown are the photomicrographs of lymphocyte proliferation in response to the TT stimulated proliferating PBMC cell clump.

(Figure 6.5D)

Figure 6.5. Direct challenge of DC with TT (3 μg) resulted in antigen processing and presentation to unstimulated PBMC. DCs were incubated with TT in the absence of PBMC, washed, and then overlain with autologous (unchallenged) PBMC as described in Methods and Materials. Shown are the PBMC proliferation in responses to: (A) control (no TT) and (B) TT challenged DC. Also shown are the photomicrographs (100x) of PBMC in unchallenged DC+PBMC cultures (C) and in co-cultures of TT-treated DC + PBMC (D). Note the foci of proliferation in (D). Cell proliferation measured by CFSE dilution following 14 days challenge with TT.
6.6 The Effect of Iron on Antigen Presenting Cells and The Impact of Iron Chelation Therapy

To determine the functional consequences of iron and iron chelators on the MPS, the effects of ferric iron (Fe$^{3+}$; ferric ammonium citrate, FAC) on dendritic cells antigen presentation immune response to tetanus toxoid antigen and the proliferation of PBMC were examined. The iron chelators tested included Desferal® (DFO) and Deferiprone (L1). PBMC were labeled with the fluorescent dye 5,6-carboxylfluorescein diacetate succinimidyl ester (CFSE) to measure cell proliferation. In addition, the effects of iron+/-iron chelators on the expression of CD83, CD80, CD86, and HLA-DR on mature DC were examined.

Importantly, iron significantly inhibited antigen presentation and PBMC proliferation. As shown in Figure 6.7A, the treatment of DC cells with 200 μM FAC for 24 hours resulted in a ~70% reduction (p<0.04) in PBMC proliferation in response to the TT antigen following 14 days of culture. To confirm that the iron-mediated immune dysfunction was due to iron toxicity, cell viability was assessed. However, 200 μM FAC treatment for 24 hours on DC does not show acute toxicity on metabolic viability assay performed as shown in Figure 6.7B. In addition, we have demonstrated DC treated with 200 μM FAC for 24 hours results in a significant amount of stainable iron by Prussian blue iron stain, compared to control cells. (Figure 6.7C and D)

However, the inclusion of iron chelators (e.g., 200-600 μM DFO or L1) restored near normal proliferation. (Figure 6.8) As L1 is a bidentate chelator, a 3:1 chelator to iron ratio was required to reverse the effect of iron to inert state. In these experiments, we have exposed 200 μM FAC pre-complexed with iron chelators L1 and DFO. As demonstrated, the adverse effect of iron in inhibiting antigen processing and presentation for TT to DC immune
response was fully restored to near normal by the 300-600 μM L1 or DFO, which confirms that iron is involved in the process of inhibiting this cell-mediated immune dysfunction.

Part of our effort is to see whether secondary iron overload patients, such as those with thalassemia, have immune dysfunction due to iron as iron-driven events give rise to anemia. While transfusion therapy corrects the anemia, it gives rise to secondary iron overload. Thus, both the primary and secondary pathology of thalassemia arise from “misplaced” iron. Removal of thalassemic RBC occurs primarily in the reticuloendothelial system (RES), via erythrophagocytosis by the mononuclear phagocytic system (MPS); these events have immunoregulatory properties, and irregular balances of iron excess may produce severe, harmful effects. Interestingly, as shown in Figure 6.7A, there is dramatic decrease of ~70% (p<0.02) in T cell proliferation in response to TT antigen presentation in DC treated with 200 μM FAC compared to positive control for TT. To our surprise, the effect of iron on APC observed in these experimental conditions was restored up to ~80% by iron chelators treated in a dose- response manner 25-200 μM L1 (p<0.02-0.04) or DFO (p<0.001-0.04). As shown in Figure 6.9, 200 μM FAC concentration causes inhibition of antigen processing and presentation in APC, but 200 μM FAC, or 200 μM DFO, or L1 does not cause acute toxicity and does not affect cell viability (p<0.001).

Similarly, CD83, an important co-stimulatory molecule expressed in DC cells, was also negatively affected by FAC in a dose (0-200 μm) dependent manner. Following 24 hours of treatment with 200 μM FAC, a ~30% (p<0.001) reduction in the mean fluorescence of CD83 was observed via flow cytometric assay. Treatment with DFO overcame the effects of iron on CD83 expression as shown in Figure 6.10. There were no significant effects of iron on CD80 or CD86, data not shown. While its actual role is unknown, CD83 is strongly
up regulated together with co-stimulatory molecules such as CD80 and CD86 during DC maturation, suggesting that it plays an important role in the induction of immune responses. Murine knockouts of CD83 show impaired T helper generation.

Figure 6.6. Iron (FAC) challenge of dendritic cells resulted in impaired antigen (TT) processing and presentation. (A) FAC-treated (200 μM; 24 hours) DC demonstrated significantly (*p<0.05) reduced stimulation of TT-mediated PBMC proliferation after 14 days relative to the untreated positive control. (B) The loss of PBMC stimulation was not due to iron toxicity as no difference in metabolic viability (i.e., MTS assay) was noted in DC treated with 0-200 μM FAC for 24 hours. Also shown are photomicrograph (200x) of (C) control and (D) FAC treated DC, stained with Prussian blue iron stain. As denoted by the arrow in (D), iron deposits are indicated by the blue stain. These results were significantly different (*p<0.05) compared with positive control cells. Shown in (A) and (B) are the mean ± SD of 3 independent experiments.
Figure 6.7. Iron chelators, when precomplexed with FAC, can prevent iron-mediated inhibition of DC antigen (TT; 3 μg) presentation. As shown, FAC treatment of DC cells alone inhibits TT-mediated stimulation of PBMC proliferation. However, challenge of DC with varying concentrations of precomplexed chelator: FAC (e.g., 100 μM DFO+200 μM FAC) demonstrated that chelation of the exogenous iron prevented the iron-mediated inhibition of TT presentation and subsequent PBMC proliferation. Importantly, the chelators alone did not affect DC presentation or PBMC proliferation. Cell proliferation measured by CFSE dilution following 14 days challenge with TT as described in Methods and Materials. The values shown are the mean ± SD of 3 independent experiments. * p<0.05 compared to positive proliferation control.
6.7 Effect of Iron on Mitochondrial Membrane Potential

Cells in hematopoietic and epithelial lineages maintain tissue homeostasis by a dynamic equilibrium balancing cell proliferation and cell death. Cell death in these lineages has been recognized as apoptotic and generally occurs after a terminal differentiation event. These physiological cell deaths are remarkably consistent in both timing and location. As we have shown, DC, antigen processing, and presentation were inhibited by iron; we also investigated other functional aspects related to cell death, such as mitochondrial functions.

As shown in Figure 6.11, mitochondrial depolarization was increased by the addition of 200 µM FAC.
µM FAC >22% compared to complete mitochondrial depolarization by CCCP control of 100%. However, iron chelation therapy by L1 and DFO 25-200 µM in dose response shows that it can reverse the mitochondrial membrane depolarization caused by iron in <5% 50-200 µM L1 or DFO treated samples. L1 and DFO 200 µM controls show that there was no effect by chelators alone on mitochondrial membrane potential (p<0.03). In addition, we have shown the qualitative effect on JC-1 aggregation in DC cells treated with 200 µM FAC and 200 µM FAC complexed with 600 µM L1 or DFO, as shown in Figure 6.12. Mitochondrial membrane potential, Δψm, is an important parameter of mitochondrial function used as an indicator of cell health. JC-1 is a lipophilic, cationic dye that can selectively enter into mitochondria and reversibly change color from green to red as the membrane potential increases. In healthy cells with high mitochondrial Δψm, JC-1 spontaneously forms complexes known as J-aggregates with intense red or orange fluorescence.

**Figure 6.10. Iron chelators (L1 or DFO) prevent iron-driven mitochondrial membrane potential depolarization.** DC-like cells were treated with 200 µM FAC for 24 hours in 1x10^6 cells per well in a 24-well plate; they were then treated with iron chelators L1 and DFO 25-200 µM for another 24 hours and stained with JC-1 cationic dye molecular probe as per manufacturers protocol. These results were significantly different (*p<0.05, unpaired t-test) compared with untreated cells. Shown are the mean ± SD of 3 independent experiments.
Figure 6.11. The protective effect of iron chelators against iron-mediated mitochondrial membrane depolarization is readily visualized microscopically. A: untreated cells (negative control) show that most of the cells had strong J-aggregation (red or orange). B: CCCP (carbonyl cyanide 3–chlorophenyl hydrazone), mitochondrial membrane disrupter treated cells (positive control) show that the majority of cells were stained green due to low ΔΨm. C: treated with 200 μM FAC. D and E: treated with 200 μM FAC pre-complex with 600 μM L1 and DFO respectively. Cells were treated with iron and chelators for 24 hours in 1x10^6 cells per well in a 24-well plate and stained with JC-1 cationic dye molecular probe as described in Methods and Materials. Photomicrographs were taken using fluorescence microscope using a broadband path filter at 200x.

6.8 Summary

While transfusion therapy corrects anemia, it gives rise to secondary iron overload in thalassemia and other hemoglobinopathies. Thus, both the primary and secondary pathology of thalassemia arise from “misplaced” iron. Removal of thalassemic RBC occurs primarily in the reticuloendothelial system (RES), via erythrophagocytosis by the mononuclear phagocytic system (MPS). These events may have negative immunoregulatory effects, and irregular balances of iron excess may produce severe, harmful effects. Our data suggests that
iron has functional consequences on the phagocytic recognition, on MPS and antigen processing and presentation to T cell proliferation. The changes observed in mitochondrial membrane potential due to iron further supports the idea that excess iron plays a key role in these cells’ apoptosis and early cell death. Also we have shown iron chelators can effectively bind and remove misplaced iron, preventing iron mediated inhibitory effects in immune recognition of antigen presenting cells to stimulate T cell proliferation. Thus, iron chelation may have therapeutic importance in the treatment of hemoglobinopathies and may prevent immune dysfunction and recurrent bacterial infection observed in these patients. Our studies resembled secondary iron overload such as in thalassemia, and suggest that the chelation of bioreactive iron within the thalassemic/sickle RBC may be an effective therapeutic intervention to treat immune dysfunction observed in thalassemia patients.
Chapter 7: Discussion

7.1 Overview

For the purpose of clarity, the discussion is divided into three sections. In the first section, “primary iron overload” of RBC and heme, and the efficacy of iron chelators to bind iron/heme in aqueous and lipid environments in order to inhibit iron-mediated oxidation are discussed. In the second section, the focus is on secondary iron overload in HepG2 and dendritic cells and the effect of iron chelation therapy. In the last section, the practical application of our findings in association with iron in transfusion medicine and its implications for the clinical practice in the Maldives is considered. In addition, we propose possible future strategies for iron chelation therapy.

7.2 “Primary Iron Overload” RBC and Heme

To date, Desferal® (DFO), Ferriprox® (L1) and Exjade® (ICL-670) are clinically used to treat an excess accumulation of iron in the body, and currently DFO is the most commonly used iron chelator for the treatment of ‘iron overload’ disorder (114). However, the disadvantages of DFO, L1, and ICL-670 involve their high toxicity and short plasma half-life (166). There are few other chelators in the research phase, such as HBED and PIH derivatives (167). As discussed in Chapter 1, for the past four decades, a large body of research has been done to improve the efficacy of available iron chelators, while reducing their toxicity and increasing their plasma retention time. In this thesis, we studied the efficacy of some LMW chelators and HMW novel chelators to bind iron in aqueous and lipid environments.

In our studies, we found that DFO, L1, and HBED bind Fe^{3+} in accordance with the
coordination chemistry and log stability constant of the various chelators. The hexadentate chelators DFO and HBED bind Fe\(^{3+}\) in a 1:1 ratio while the bidentate chelator L1 binds Fe\(^{3+}\) in a ratio of 1:3, as shown in Figure 4.1. These findings are important, as the stability of the iron/chelator complex depends on the number and size of chelate rings formed in the Fe\(^{3+}\) chelator complex (110). When selecting iron chelators for biological and clinical applications, the properties governing metal selectivity and ligand-metal complex stability are paramount. Especially when using a bidentate chelator such as L1, there can be bioavailable iron left over, which may lead to oxidative damage if the chelator to iron ratio is not maintained (168).

Looking for ways to improve the efficacy of existing LMW chelators, we also conjugated biocompatible polymers, such as polyethylene glycol (140), to various LMW monomers, including previously reported DFO modification by starch-based polymers (137). For example, ICL-670, a tridentate ligand, is extremely insoluble in water, but in our studies, we have found that it is fully soluble when conjugated to PEG (SK-1) (136). In addition, UV spectral studies showed that SK-1 has the same efficacy to bind iron in aqueous conditions when compared to its monomer. Similarly, we have conjugated PEG with DFO (P-DFO), and used commercially available starch conjugated to DFO (S-DFO), both show efficacy comparable to monomer DFO in iron binding efficiency. We further studied the cytotoxicity of our novel HMW chelators, P-DFO and S-DFO; ~90% cell survival was observed compared to DFO alone (140). Interestingly, P-DFO and S-DFO have comparable patterns of thermal stability and decomposition in thermogravimetric analysis. The data indicates that polymer-based chelators might have potential advantages, similar to that of the starch-conjugated modification of DFO (137,143). Dextran and hydroxyethyl starch-based
modification of DFO has been reported to exhibit significantly improved plasma retention and substantially lower acute toxicity levels ($LD_{50}>4000$ mg DFO equivalents/kg in mice) than free DFO ($LD_{50} \sim 250$ mg/kg) time in animal studies (137). To date, we do not have P-DFO based in vivo studies, but comparison is possible with the wide range of PEG based in vitro and in vivo studies reported by Scott and others in cells and tissues (144,145,169). We believe P-DFO with certain ranges of polymer length and molecular weight may have advantages over S-DFO in the design and formulation of a new class of HMW chelators (140). The new formulation of S-DFO is designed to have shorter HMW fragments that in circulation can be cleaved by serum amylase (143). The presence of PEG linear polymer structures and a resulting increase in the Stoke’s radius of the bound DFO might improve vascular retention, making it more suitable for systemic vascular applications (170). Even though HES has been used for hemodilution and plasma expanders, unwanted side effects of coagulation have been well documented (171).

Treating normal RBC with PMS stimulates intracellular oxidation; our combination chelation treatment has shown an additive effect to inhibit RBC lysis due to intraerythrocytic iron-mediated oxidative reactions (172). These results encourage us to support our hypothesis that combination iron chelation therapy may have therapeutic advantages. The pathophysiology of thalassemia has been discussed earlier; Scott and others have shown that while the primary pathology of thalassemia remains due to point mutations, iron-mediated intraerythrocytic oxidative damage further enhances early hemolysis, and cell death leads to severe anemia (33,43,173). As we have shown in Figure 4.5, L1 and HBED, both cell permeable LMW chelators, and S-DFO, a cell impermeable HMW chelator, combination treatment have shown a 30-33% additive effect.
We have also hypothesized that erythrophagocytosis of thalassemic RBC may have an immunosuppressive effect by inhibiting normal macrophage functions. Our experiments have shown that for PBMC, tetanus toxoid or *streptococcus mutans* initiated antigen processing and presentation is inhibited by either hemin or PMS oxidized RBC. The data are comparable to that of the pathology of thalassemia patients, as most erythrocytes are primarily cleared by splenic sinusoids via erythrophagocytosis by the mononuclear macrophage system. Perhaps the recurrent bacterial infections frequently seen in thalassemia major patients are due to the iron-mediated immune dysfunction of MPS (22,174).

To determine if the selected iron chelators bind and effectively remove iron or heme, we studied the inhibitory effect of iron-mediated hemoglobin oxidation in hemolysate as well as of purified hemoglobin. With no added iron in hemolysate, there is no hemoglobin oxidation observed; in normal physiology, however, there is about 1.5-3% auto-oxidation in hemoglobin (175). Interestingly, in a dose response manner 0-250 µM Fe$^{3+}$ shows severe hemoglobin oxidation, but inclusion of iron chelators DFO, HBED, L1, S-DFO and our novel chelator P-DFO fully inhibited the Fe$^{3+}$ mediated hemoglobin oxidation normally observed, which is near to normal in dose response manner. However, purified hemoglobin does not show significant hemoglobin oxidation with added iron, but the inclusion of a reducing agent, such as reduced glutathione (GSH), has shown a similar kind of hemoglobin oxidation to that seen in hemolysate (15). In addition, we observed higher concentrations of GSH >1 mM alone without added iron, and also oxidized purified hemoglobin, suggesting either there is residual iron or that GSH acts as a pro-oxidant. Nevertheless, the chelators DFO, HBED, L1, S-DFO, and P-DFO also inhibited Fe$^{3+}$/GSH driven hemoglobin oxidation in a dose response manner.
Usually a hexadentate chelator that is fully saturated with iron such as the Fe$^{3+}$/DFO complex is very stable in aqueous conditions. To our surprise, we observed that even though Fe$^{3+}$ mediated hemoglobin oxidation was inhibited by DFO within minutes of a addition, the prolonged incubation of Fe$^{3+}$/DFO complex itself caused oxidation and methemoglobin formation. However, Fe$^{3+}$/S-DFO has shown very significant inhibitory effects in the days after incubation. This protective effect again confirms the advantages of S-DFO and also shows that HMW chelators have a greater protective effect while maintaining their functional characteristics for a longer time.

To observe whether the effect of iron binding agents can diminish the oxidative potential of iron, membrane peroxidation was examined by TBARS formation due to lipid peroxidation (137). RBC membrane ghosts were exposed to iron$^{+}$/chelators L1, DFO, and P-DFO challenged with reducing agent cumene hydro peroxide and showed a significant inhibition of TBARS formation in a dose response manner compared positive control for iron (137).

### 7.3 Secondary Iron Overload

Because of regular transfusions, thalassemia major patients are subject to secondary iron overload in tissues and organs (44, 99). These patients often die due to organ failure from secondary diseases, such as diabetes, liver cirrhosis, and cardiac failure (99). We studied an *in vitro* cell culture model of HepG2 cells, which resembles secondary iron overload in thalassemia and sickle cell disease (176). HepG2 cells were iron loaded with FAC and then treated with iron chelators. Increased concentrations of iron $>2$ mM showed their adverse effect on metabolic activity of cells, but $<1$ mM FAC did not show a detrimental effect. However, 200-500 μM FAC successfully accumulated iron in cells, while
maintaining their function (177). In these experiments, we observed microscopically from Prussian blue iron stain a qualitative decrease in iron with L1 and S-SDFO combination chelation therapy after 12-48 hours of treatment. In addition, there was a significant decrease in cellular ferritin after 12-24 hours of treatment, showing additive effects. Interestingly, there was ~30% significant synergistic decrease in total iron in ferrozine chelatable iron in cell pellet. The data are promising, supporting the hypothesis that combination chelation therapy with LMW cell-permeable chelators, such as L1 and cell impermeable chelators such S-DFO, may have therapeutic advantages, compared to either of the chelators alone.

We also studied the effect of iron on ultrastructures and organelles in these cells with transmission electron microscopy. Significant changes in ultrastructures; especially lysosomes and mitochondria with electron-dense siderosomes, in 200-500 μM FAC treated cells were observed (151). To our surprise, these combination chelation therapies of DFO and S-DFO showed significant improvement compared to control cells, because the electron dense siderosomes disappeared. Perhaps these organelles findings may be future therapeutic targets for iron chelation, as lysosomal accumulation of iron and the effect of iron chelation has also been previously reported (165).

Another important aspect is the hypothesis that the erythrophagocytosis of abnormal RBCs, such as thalassemic and sickle cells, may have immune suppressive effects. It is well known that thalassemia patients have common recurrent bacterial infections, which could be because of their dysfunctional immune competence due to excess iron (178). There are some published data from thalassemia patients showing that important immune regulatory markers are either up regulated or down regulated (93). To support our hypothesis, we studied immune responses using professional antigen presenting cells (dendritic cells) derived from
human PBMC (179). We noted dramatic decreases in T cell proliferation, immune response to tetanus toxoid-mediated antigen processing, and presentation in iron-exposed dendritic cells. Interestingly, the iron chelators DFO and L1 eliminated the iron-mediated inhibition of the immune response. Also, we noticed that some of the surface markers of mature dendritic cells, such as CD83, are also affected and restored with iron chelation (180). To see the functional aspects of DC, we studied mitochondrial membrane potential, one of the early signs of cell death and apoptosis (181). Our studies have shown that iron-treated DC increases mitochondrial membrane depolarization and iron chelators L1, and DFO dose dependently reverses the depolarization effect seen in iron control cells. The data supports our hypothesis that misplaced iron in MPS has negative immune regulatory effects that may be the reason that thalassemics are prone to recurrent infection; improved iron chelation therapy may prevent these infections (150).

7.4 Future Directions

7.4.1 A Practical Application

There are few platelet transfusions in the Maldives because of the risk of microbial infections. Can iron chelators reduce the risk of microbial infections in platelets? We hypothesize that blood products such as platelets can be stored for a longer period of time with the inclusion of iron chelators to inactivate bacterial growth due to residual iron from RBCs in platelet bags. Another area where iron plays an important role in transfusion medicine is in the production of blood products. The shelf life of RBC concentrates at 4 °C has a low risk of microbial growth, but platelet concentrates at 22 °C have a high risk of bacterial growth because of residual iron from RBCs in the plasma (182) (Figure 7.1) In the
USA, 1.5 million platelet products are used each year and in Europe the number reaches 2.9 million (183). However, even in North America and Europe, it remains a challenge to maintain the quality of platelet supplies due to the delicate balance of platelet metabolism; as a result, the handling and storage of platelets is still an arduous task for many researchers in this field. In underdeveloped countries, the situation is worse. In the Maldives for instance, platelet concentrates are not currently prepared. We believe that the inclusion of iron chelators in blood products such as platelet bags may have a high potential to reduce the risk of bacterial contamination when utilized in underdeveloped countries, such as Maldives. Some preliminary experiments, I performed on bacterial growth show that DFO can inhibit *staphylococcus epidermidis* growth, while the addition of 100 μM Fe³⁺ restores bacterial growth. *(Figure 7.2)* The data supports the release of iron from residual RBC, which may be present in the plasma of platelet bags; if there are bacteria, such as *staphylococcus epidermidis*, a normal flora of skin, they might contaminate plasma and start growing in platelet bags, as the temperature is favorable for bacterial growth.
Figure 7.1. Scheme of platelet concentrate bags, iron release from residual RBC, and risk of contamination of bacteria and restoration by iron chelators. As shown limited bioavailability of iron from residual RBCs in platelet bags, may enhance bacterial growth with time, inclusion of iron chelators inactivate bacterial growth. (Courtesy of Dr. Scott)

Figure 7.2. Bacteriostatic effects of iron chelators. Chelation of bioavailable iron prevents bacterial growth as detected by spectrophotometric analysis of bacterial growth curves, A shows an iron chelator DFO [5-500 μM] fully inhibited *staphylococcus epidermidis* growth in nutrient broth. In B, the addition of 100 μM Fe³⁺ after 24 hours of incubation restores bacterial growth.

7.4.2 Proposed Ideal Future of Iron Chelation Therapy and Its Implications in Transfusion Medicine

As we have hypothesized an alternative therapy for thalassemia where the chelation of misplaced iron from within the RBC can slow or prevent the injury to thalassemic RBC, resulting in improved RBC survival within the circulation and tissues, as well as the protection of immune competency. We also believe that iron chelation therapy can improve transfusion medicine safety by chelating redox-available iron. To accomplish this improvement, we propose a *novel shuttle chelation system* as discussed in Chapter 1.
Prolonged circulation time of the RBC would decrease the degree of anemia in a significant number of patients, potentially preventing life-long transfusion therapy. In addition, the shuttle chelation system can be used to treat secondary iron overload (e.g., liver) arising from transfusion therapy. It is our belief that improved iron chelation therapy will improve tissue metabolism and the thalassemic individual’s quality of life. Iron chelation can effectively bind and remove free and complexed iron/heme, preventing both redox-driven damage and immunosuppression. We also believe that iron shuttle chelation can be used to detoxify blood products, such as platelets, in underdeveloped countries such as the Maldives where there are inadequate resources to store and supply safe blood.

We strongly believe the future treatment of transfusion-dependent thalassemia will be with less toxic HMW polymer-based and higher iron log stability constant chelators, such as P-DFO or S-DFO, that may be administered twice monthly or as weekly injections in combination with LMW chelators, such as Exjade® or L1 the oral chelator, improving the quality of life of thalassemia major patients. (Figure 7.3) With mild to moderate anemia, humans can survive if we can increase hemoglobin levels several fold by reducing intraerythrocytic iron-mediated redox reactions; perhaps we may prolong the life of RBC reducing anemia to the extent that regular life long transfusions are no longer necessary.
Figure 7.3. Scheme of proposed future iron chelation therapy. Iron shuttle chelation therapy administered by using daily oral iron chelators and twice-monthly injections of HMW chelators. The clearance can be via renal system and intestine. In this way of treatment plasma retention time of iron chelators will increase and maximum benefit can be achieved to reduce iron accumulation.

7.5 Conclusions

The pharmacological interception of intraerythrocytic iron in β-thalassemic RBC may substantially prevent or diminish iron-dependent injuries to the erythrocyte and its progenitor cells. The inhibition of iron-mediated damage may increase “effective erythropoiesis” (red cell mass) and may subsequently reduce the necessity of blood transfusions. Moreover, improved iron chelation for transfusional iron overload (secondary iron overload) will improve immune competence and reduce organ failure and death. Thus, the development of interventions that improve effective iron chelation while minimizing chelator toxicity may have significant therapeutic utility in developing countries, such as the Maldives.
References


