CHAPTER I
INTRODUCTION

1.1 Statement and significance of the problems

Iron is essential for many cellular processes such as proliferation, energy production and oxygen transport. During years of growth iron absorption must exceed iron loss by approximately 0.5 mg per day to maintain a body iron concentration of about 60 parts per million. An adult male (70 kg) has a total body iron of about 4 grams, which it remains constant throughout his life by a balance between absorption and loss of iron in the human body. Physiologically, 65% of the total iron is bound to hemoglobin, 10% is constituents of myoglobin, cytochromes and iron-containing enzymes, and 20-30% is bound to plasma transferrin mainly, and to intracellular ferritin and hemosiderin. In healthy state, iron is "kept safe" by binding to the transferrin and ferritin. In iron overload like thalassemias, iron-binding capacity of transferrin is markedly increased in both plasma and cellular compartments; consequently, non-transferrin bound iron (NTBI) and labile cellular iron (LCI) appear in plasma and cytoplasm respectively. The NTBI and LCI potentially catalyze formation of reactive oxygen intermediates (ROI) via Haber-Weiss and Fenton reaction in erythrocytes, endocrine glands, pancreas, liver and heart. Some reactive oxygen specie (ROS) such as hydrogen peroxide is required for killing microorganisms in neutrophils, an excess amount can be toxic. The ROI and free radicals can induce damage and dysfunction of the vital organs (Britton et al., 2002).

Thalassemia patients with iron-overload suffer from anemia, pituitary glands malfunction, type II diabetic mellitus, liver fibrosis and cardiac arrhythmia (Beutler et al., 2003). Eventually, they will be fetal unless suitable iron chelation therapy is managed. Importantly, 60-70% of the β-thalassemia major patients die of congestive heart failure. Normally, transferring-bound iron is taken up into the cells via transferrin receptors-dependent mechanism under strict iron homeostasis. Within the cells, iron enters the transit pool (also called labile iron pool, LIP), which serves as a crossroad of cell iron metabolism, for further utilization and storage in ferritin. Nevertheless, the NTBI uptake is independent of the transferrin receptors and up-

regulated in cardiac myocytes and hepatocellular carcinoma cells or HepG2 cells (Beutler et al., 2003; Randell et al., 1994), rat primary hepatocytes (Barisani et al., 1995). Possible mechanisms of the NTBI uptake could be divalent metal transporter (DMT) (Garrick et al., 1999; Randell et al., 1994), L-type calcium channel (LTCC) (Oudit et al., 2003; Oudit et al., 2006), and Zip14 (a member of SLC39A zinc transporter family (Liuzzi et al., 2006). The LIP is redox-active and highly increased in iron overload condition. It plays a role in free radicals generation and is the main target of chelators. Presumably, LIP and ROI levels follow similar "rise and fall" patterns as a result of changes in iron import versus iron chelation, or ferritin degradation versus ferritin synthesis.

Hepatic stellate cells (HSC) referred as Ito cells, fat-storing cells or lipocyte locate between parenchymal cell plates and sinusoidal endothelial lining cells of the hepatic lobule. In chronic injury, activated HSC display proliferation and fibrogenic myofibroblast phenotype, alpha-smooth muscle actin (α-SMA), enhanced contractibility and high synthesis of extracellular matrix (ECM) component, especially collagen (Arthur, 1996). Hepatic fibrosis represents the liver's wound healing response and is characterized by accumulation of interstitial matrix, or scar. Increased synthesis of collagen leads to fibrosis and eventually cirrhosis. Activation of HSC is a key event in fibrogenesis and indicates early changes in cellular phenotype, progressive injury and activation in terms of growth characteristics, response to soluble mediators, inflammatory signaling, and apoptotic potential (Friedman, 1999). The cellular changes include increasing and enlargement of rough endoplasmic reticulum, ruffled nuclear membrane and appearance of contractile filaments. The initiation phase is due to paracrine stimuli from neighboring injured hepatocytes, Kupffer cells, sinusoidal endothelial cells, platelets, and infiltrating inflammatory cells. Some of hepatocytes and Kupffer cells promote HSC activation by producing lipid peroxides.

Increased hepatic iron concentration (HIC) in patients with transfusional β-thalassemia and non-alcoholic steatohepatitis (NASH) and over-consumption of alcohol is associated with liver toxicity, fibrosis, cirrhosis, and hepatocellular carcinoma in humans (Asare et al., 2006; Fletcher et al., 2003; George et al., 1998; Stal et al., 1995). While most iron circulates in blood as transferrin-bound iron, non-

transferrin-bound iron (NTBI) also becomes elevated and contributes to toxicity in the setting of iron overload. A recent study has shown that NTBI could be a tumor promoter in T51B rat liver epithelial cells and cell cycle disregulation contributes to tumor promotion by NTBI (Messner and Kowdley, 2008). Hepatic histological examination showed that chronic active hepatitis C and the accumulation of iron would be the major causative factors of liver fibrosis (Tondury et al., 1998). Iron-overload atients with primary hemichromatosis showed raised serum alanine aminotransferase (ALT) activity (Bhavnani et al., 2000). Intraperitoneal injection of iron dextran increased amounts of hemosiderin and ferritin in cytosol of liver of iron-loaded rats. Measurements of liver iron concentration by means of semi-quantitative inductive device (SQUID), R2*-magnetic resonance imaging (MRI) and Pearl's staining are usually used to evaluate efficacy of iron chelation therapy (Fischer et al., 2005; St Pierre et al., 2005; Thakerngpol et al., 1988). DFO and DFP chelation therapy can decrease levels of ferritin and NTBI in serum and amount of iron burden in liver of hereditary sideroblastic anemia (Meo et al., 2006).

Desferrioxamine (DFO), deferiprone (DFP) and deferasirox (DFX) are promising iron chelators that are widely used for treatment of iron-overload patients (Porter, 1997). Main target organs of iron chelation therapy are liver and heart which accumulate a large amount of iron in cytosolic ferritin, hemosiderin and transitory pool (Britton et al., 2002; Cappellini, 2008; Cappellini and Piga, 2008). Effectiveness, cost, compliance, life quality and side effects of the chelators are relevant considerations. DFO and DFP were able to decrease liver iron concentration in transfusion-dependent patients with β-thalassemia major (Nielsen et al., 1995). Interestingly, epigallocatechin 3-gallate (EGCG) and epicatechin 3-gallate (ECG) are major polyphenolic constituents in green tea (GT), which show strong anti-oxidant and free-radical scavenging activities. Their beneficial effects can protect cells from free-radical imbalance. Studies about anti-oxidative and metal-chelating activities of GT polyphenols both in vitro and in vivo are interesting and attractive. One or more major catechins from the GT extract could stoichiometrically bind ferric ion to form a redox-inactive iron-polyphenol complex. Combination effects of the GT catechins potentially can protect vital biomolecules from oxidative damage. Logically, it may

have a capacity to chelate excess iron in iron-overloaded condition and play important protective roles in this un-pleasured condition.

Literature Review

1.2 Iron metabolism and homeostasis

Iron plays a crucial role in vital biochemical activities, such as oxygen sensing and transport, electron transfer, and catalysis (Aisen et al., 2001). Biological functions of iron are based on its chemical capabilities including forming coordination complexes with organic ligands, and favorable redox potential to switch between ferrous (Fe^{2+}) and ferric (Fe^{3+}) states (Papanikolaou and Pantopoulos, 2005). Physiologically, iron is totally bound to transferrin in plasma and ferritin in tissues; therefore, there is not appreciable concentration of 'free iron' for chemical catalysis. In thalassemia patients their plasma transferrin is fully saturated, any released and absorbed iron is immediately chelated by physiological ligands such as citrate, phosphate and album to form non-transferrin bound iron (NTBI) and labile plasma iron (LPI). The NTBI and LPI are subsequently taken up into liver cells and heart cells and will appear as labile iron pool (LIP) in cytoplasm (Nelson and McCord, 1998). The redox iron can participate in generation of superoxide ($O_2^{\bullet -}$) and convert hydrogen peroxide (H_2O_2) to hydroxyl radicals ($HO^{\bullet}$) collectively known as "reactive oxygen species" (ROS) *via* Haber-Weiss and Fenton reactions. The ROS can attack biomolecules (e.g. lipids, proteins and DNA) which are essential cellular constituents (Gutteridge et al., 1982). Under excess free radical condition (called oxidative stress) tissues and vital organs can be damaged by action of the existing ROS, leading to tissue damage and organ dysfunctions.

Living organisms are protected from iron-mediated oxidative damage by iron-sequestering proteins and anti-oxidative defense mechanism (Emerit et al., 2001). Male adults normally contain 35-45 mg iron per kilogram body weight (Andrews, 1999). Premenopausal women have lower iron stores as a result of their menstruation. Normally, 1-2 mg of iron enters and leaves the body each day. Dietary iron is absorbed by duodenal mucosal cells, bound to plasma transferrin in blood circulation and accumulates within cells in ferritin. Most of the iron in the body is incorporated into hemoglobin in red cells. Approximately 10-15 percent of the iron is present in

myoglobin in muscle fibers, in some enzymes and cytochromes in hepatic tissues. Iron is also stored in reticuloendothelial (RE) macrophages, which they provide most of the usable iron by degrading hemoglobin in senescent erythrocytes and reloading ferric ion onto transferrin for delivery to cells.

Cellular iron homeostasis is obtained by opposite regulation of ferritin and transferrin receptors (TfR) synthesis (Aisen et al., 2001; Richardson and Ponka, 1997). The mechanism involves specific mRNA sequences named as iron responsive element (IRE) that forms stem loop structures with an affinity for iron regulatory proteins (IRP). Under cytosolic iron depletion, affinity of the IRP for the IRE is greater than for the iron.

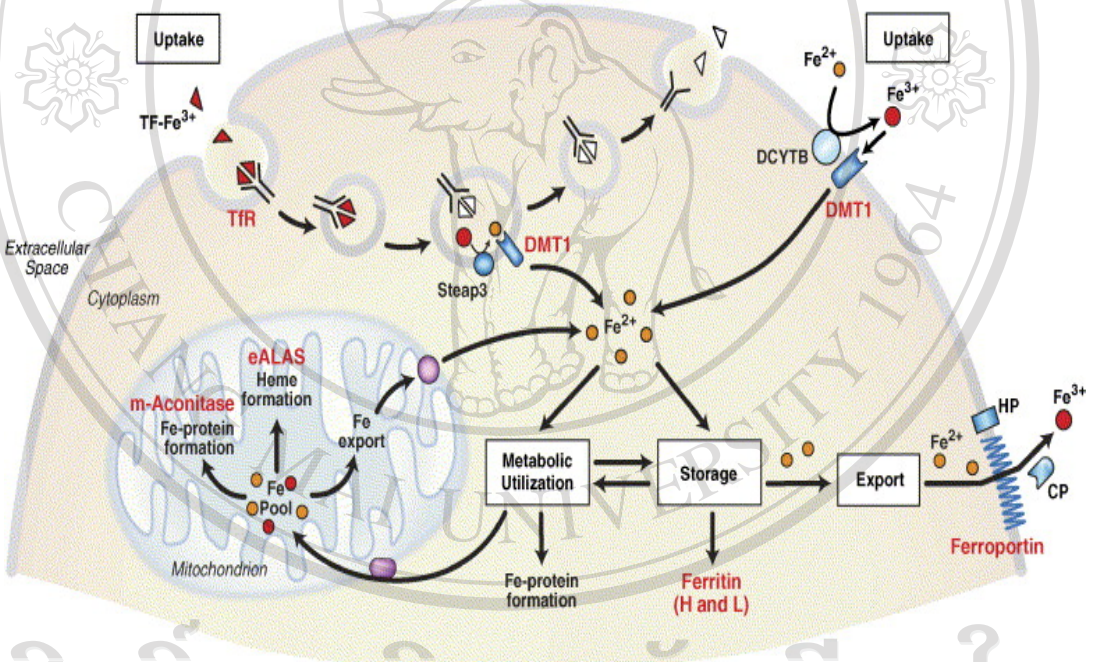

Figure 1-1 Mammalian iron homeostasis (Wallander et al., 2006)

In this case translation of ferritin mRNA to ferritin polypeptide chain is suppressed, whereas TfR mRNA is stabilized and synthesis of TfR protein increases on the opposite end. In iron overload the affinity of IRP for IRE decreases, the ferritin synthesis is stimulated, and the TfR synthesis is suppressed. This mechanism controls iron supply to the cells and assures that any excess iron is stored safely in ferritin (Harrison and Arosio, 1996).

1.3 Iron overload in thalassemias

Iron overload commonly results from inherited and acquired causes such as increased iron absorption and multiple blood transfusions (Siah et al., 2005). Excess iron accumulated in organs and tissues eventually leads to clinical manifestations including diabetes, skin pigmentation, hypogonadism, arthritis, liver cirrhosis and cardiomyopathy. β-Thalassemia is a common secondary hemochromatosis accompanied with ineffective erythropoiesis and arises in childhood and adults (Beutler et al., 2003). Blood transfusion can add stoichiometrically iron (around 1 mg Fe/ml of red blood cells, 0.47 mg Fe/ml of whole blood, or 1.16 mg Fe/ml of pack red cells) to the body. Examples of inherited defective β-globin gene(s) are β-thalassemia major (severe anemia with regular transfusion dependence), β-thalassemia intermedia (mild anemia with intermittent blood transfusion) and β-thalassemia HbE (moderate anemia with intermittent blood transfusion). Splenectomized β-thalassemia major patients receive about 140 mg transfused Fe/kg of body weight (0.4 mg transfused Fe/kg) to maintain a mean hemoglobin level of 12 g/dl. Transfusion requirement in unsplenectomized patients is generally higher than splenectomized patients and may contribute to an increased iron loading. Added with dietary iron absorption (1-4 mg per day) the iron will accumulate 28-35 mg/day in splenectomized β-thalassemia patients (Fiorelli et al., 1990; Vatanavicharn et al., 1983).

Oxidative stress is a consequence of overproduction of free radicals and can deteriorate functions of vital organs. Thalassemia patients are prone to oxidative stress and suffer from clinical complications. Organ malfunction and short life-span red cell are usually caused by intracellular excessive ROS and iron deposit. Reactive oxygen radicals are thought to involve in pathophysiology of thalassemia. Release of free iron from α-chains in β-thalassemic red blood cell (RBC) can itself initiate redox reactions which simultaneously deplete level of intracellular GSH and accelerate RBC destruction (Scott and Eaton, 1995). The RBC destruction in bone marrow of thalassemia patients partially increases apoptosis of erythroid precursors and relate to membrane damage.

During iron overload, blood stream is the first compartment where defensive systems against iron toxicity may be overpowered (Pietrangelo, 2002). Increased levels of serum iron (SI), transferrin saturation (TS) and serum ferritin (SF) are early indicators of iron overload (Han et al., 2004). Non-transferrin bound iron (NTBI) is detectable in plasma and catalyzes ROS formation (Breuer et al., 2000; Cabantchik et al., 2005). The NTBI, a potential toxic form of iron, can be subsequently taken up into liver and heart cells *via* a specific mechanism. Labile plasma iron (LPI) is a component of the NTBI which is redox active and susceptible to chelation (Pootrakul et al., 2004). Labile iron pool (LIP) or labile cellular iron (LCI) is a chelatable and redox-active iron, which is transitory and served as a crossroad of cellular iron metabolism (Kakhlon and Cabantchik, 2002). Cell damage associated with iron overload has been attributed to the emergence of level of LIP that promote the ROS generation exceeding cellular defense capacities (Hershko et al., 2005). In β-thalassemia major, the redox iron is potential to damage vital organs such as heart, liver and endocrine glands. Main objective of chelation therapy is to remove the forming and persisting NTBI as well as LPI in plasma, and LIP in the cells (Glickstein et al., 2005).

Free iron, plasma NTBI and intracellular LIP, plays a crucial role in the generation of ROI, in particular hydroxyl radical ($HO^{\bullet}$) which is harmful to the cells through reactions such as lysosomal fragility, catalysis of lipid peroxidation and increased collagen synthesis. Liver tissue from thalassemia patients shows markedly accumulated iron and fibrosis. Severe degrees of hemosiderosis and fibrosis are found in hepatic parenchymal cells and Kupffer cells in most case (Thakerngpol et al., 1988; Vichinsky et al., 2005). Major pathologic effects of chronic hepatic iron overload are fibrosis and cirrhosis, porphyria cutanea tarda, and hepatocellular carcinoma (Bonkovsky, 1991).

Beta thalassemia (β-thalassemia) is characterized by reduced (β^{+}) or absent (β^{0}) synthesis of the β-globin chain. In contrast to the α-thalassemia, most of the mutations resulting in β-thalassemia are caused by point mutatin (single base substitution) or small deletion and insertion. At present, more than 50 mutations have been characterized. Mutations causing β-thalassemia result in deficit of β-globin production that ranges from minimal (β^{+}-thalassemia) to a complete absence (β^{0}-

thalassemia). β^0-thalassemia or Cooley anemia is much more common than β^+-thalassemia. In Thailand, the most common mutation is the 4 bp deletion is codon 41/42 which accounts for 45% of β-thalassemia in Thailand. The most common β^+-thalassemia in this region is HbE (14). It is a β-globin variant resulting from substitution of Glu→Lys at codon 26. The G→A mutation in the codon 26 also activates an adjacent cryptic splice site located at codon 24 to 27 leading to an alternative splicing which produces no β-globin chain whereas the normal spliced mRNA which contains the exon 1 mutation at codon 26 produces β^E-globin.

β^0-thalassemia is usually more severe than β^+-thalassemia. Howover, a wide range of β-globin production are observed in β^+-thalassemia. Some mutation may produce little amounts of β-globin and the phenotype is similar to β^0-thalassemia, for example the C→T in IVS II nt 654 (15, 16). Interaction betaween HbE and mild β^+-thalassemia such as A→G at position –28 or A→G in codon 19 usually results in a mild thalassemia phenotype. Clinically, β-thalassemia is classified into three major groups:

β-thalassemia minor. This group includes all the heterozygotes, or thalassemia traits who are asymtomatic. Some homozygous, compound and doubly heterozygous subjects are also symptom-free, such as homozygotes for HbE. Their Hb levels are normal or near normal. They have no jaundice, hepatosplenomegaly or iron overload, but some develop iron deficiency.

β-thalassemia intermedia (TI). This group consists of β-thalassemia diseases with mild-to-moderate anemia; the average Hb level in the steady state is 7-8 g/dl. TI is usually associated with mild-to-moderate jaundice and hepatosplenomegaly; patients with high Hb levels have no definite gross abnormalities in physical development and no thalassemic facies. Generally, the patients have mild symptoms or are symptom free, but complications do occur. Iron overload is always demonstrated by raised plasma ferritin levels. Normally, patients with β-thalassemia intermedia do not require blood transfusions except when they develop infections which exacerbate the anemia. Iron chelation may not be necessary in very mildly affected patients, but should be considered in more severe cases. Two major genotypes give rise to β-thalassemia intermedia phenotype in Southeast Asia.

β-thalassemia disorders: Numerous β-thalassemia genes, either in the homozygous state or in compound heterozygosity with one of β^0-thal genes, menifest as β-thalassemia intermedia. The interaction of α-thalassemia gene or a high HbF gene with severe β-thalassemia may alleviate the imbalance of globin chains and modify the degree of anemia.

β-thalassemia/HbE disease: Approximately 50% of β-thalassemia HbE patients manifest as TI, while the remainder have severe thalassemic disease.

β-thalassemia major: This group shows severe anemia and associated symptoms. Hb level are usually 6 g/dl or lower. Untreated, the patients die in the first two decades of life. β^0-thalassemia HbE disease is the most common cause of β-thalassemia major in Southest Asia. Their Hb levels in the steady state range from 3 to 13 g/dl with an average of 7.7 g/dl.

Pathology of β-thalassemia

The underlying pathophysiology of β-thalassemia relates to a quantitative deficiency of functional β-globin chains, which leads to an imbalanced globin chain production and an excess of α-globin chains. The latter aggregate in red cell precursors, forming inclusion bodies thalassemia causes mechanical damage and their premature destruction in the bone morrow. Red cells that survive to reach the peripheral circulation are prematurely destroyed in the spleen. Anemia in β-thalassemia thus results from a combination of ineffective erythropoiesis, peripheral hemolysis and an overall reduction in Hb synthesis. A direct effect of the anemia is the increased production of erythropoietin, which leads to intense proliferation and expansion of the bone marrow with the resulting skeletal deformities. These secondary complications of bone disease, splenomegaly, endocrine and cardiac damage can be related to the severity of anemia and the iron leading that results from the increased gastrointestinal absorption and the blood transfusions .

Iron overload occurs without exception. The skin is darkend and iron deposition occurs in bone marrow, liver, spleen, heart, pancreas, and elsewhere (Pootrakul et al., 1981). Arrhythmia is not frequently encountered as in homozygous β-thalassemia. Liver fibrosis and other signs of cirrhosis from iron overload is common (Sumida et al., 2009; Wu et al., 2006). Diabetes mellitus secondary to iron deposition in the pancreas develops if patients live long enough. As iron overload is a

constant complication of thalassemia and iron is a strong oxidant, suppression of body antioxidants such as vitamins C and E is a usual finding in thalassemia patients.

1.4 Estimation of Tissue Iron

Serum ferritin

This is a useful technique for assessing changes in body iron, although the absolute level is an imprecise measure of body iron. This is partly because inflammation—for example, hepatitis C—raises the level, while vitamin C deficiency lowers it, both frequent complications of TM. Most studies have found a wide range in liver iron at any given serum ferritin level. The Thalassemia International Federation guidelines (Abe, K. et al., 2007) recommend maintaining serum ferritin levels around 1000 µg/L; nevertheless, levels below this may be associated with cardiac complications. One study in TM patients receiving DFO found that those with at least two-thirds of serial serum ferritin estimations less than 2500 µg/L had significantly less cardiac disease than those with higher levels (Andrews, N.C. et al., 1999).

More recently, a level consistently below 1500 µg/L was found to be associated with few complications in 32 patients with TM followed for approximately 15 years. When effective chelation therapy is initiated, the serum ferritin falls more rapidly than body iron. This may happen partly because of improvement in liver function and partly because serum ferritin may reflect predominantly reticular endothelial iron rather than parenchymal iron in the liver and other organs.

Liver iron

Liver iron has been described as the "gold standard" for determining body iron and has been recently shown to correlate with total body iron stores.[7] It can be measured chemically after liver biopsy (which can be inaccurate because of fibrosis, cirrhosis, or uneven distribution of iron) or noninvasively by the superconducting quantum interface device (SQUID) (available in only a few centers) or by magnetic resonance imaging (MRI). Studied 59 TM patients who were more than 7 years old and died had liver iron concentrations >15 mg/g dry weight has been subsequently regarded as an index of high risk of death from cardiac disease. More recently, Angelucci et al. have shown that this level is also associated with liver fibrosis and

cirrhosis. The level of 7 mg/g is the upper limit found in carriers of genetic hemochromatosis. For levels between 7 and 15 mg/g, Angelucci et al found no evidence of liver damage except in patients who had hepatitis C and were messenger RNA positive; the combination of iron overload and hepatitis C infection is particularly damaging to the liver.

The value of liver iron, whether >15 mg/g or in the range of 7 to 15 mg/g, as a predictor of cardiac iron has recently been questioned. MRI data using the T2* technique and spin-echo have shown no correlation between cardiac and liver iron, although other MRI techniques, possibly less sensitive and accurate, have shown such a correlation.

Cardiac iron

Direct measurement of cardiac iron by endomyocardial biopsy of the right atrium is inappropriate since iron locates mainly to the myocardium of the ventricles. The recent development of a reproducible, sensitive, and accurate indirect measure of cardiac iron using the MRI T2* technique has provided substantial important new data. A T2* value less than 20 ms has been found to correlate with the presence of cardiac dysfunction, detected by echocardiography, 24-hour monitoring, or the need for cardiac therapy. It is also valuable for monitoring changes in cardiac iron during intensive chelation therapy.

Non-transferrin-bound iron

In severely iron-loaded patients, non-transferrin-bound iron (NTBI) is present in plasma. It occurs in 80% of patients with TM and represents a highly toxic species causing tissue iron loading. NTBI is also found in patients receiving chemotherapy or undergoing heart bypass operations and those having other conditions in which large amounts of iron from hemoglobin breakdown are released into the circulation. NTBI is removed by administration of DFO or DFP but reappears rapidly unless body iron burden is substantially reduced.

Urine iron excretion

Iron excreted in response to a single dose of DFO or deferiprone has been taken as an index of body iron burden. It will vary, however, with the dose of chelator used and, for DFO, whether vitamin C is also given and with the hemoglobin level.

There is also considerable day-to-day variation, even with apparently the same conditions.(Aisen, P. et al., 1999).

. Nevertheless, in one recent study it has been found to correlate closely with cardiac iron measured by MRI.

Estimation of iron-induced tissue damage

In addition to measuring iron status, it is important to assess the function of the heart, liver, and endocrine glands, the organs particularly damaged by iron overload. Early detection of cardiac dysfunction is especially important so that increased chelation therapy can be instituted before cardiac damage is irreversible. Once the patient has started chelation therapy, it will also be necessary to monitor for potential side effects of the iron chelator being used

1.5 Models of thalassemic mouse

The mouse β-globin gene cluster on chromosome 7 has four functional β-globin genes: β*h1*, an early embryonic globin gene; εγ2, a late embryonic globin gene; and two adult globin genes, *b1* (β major) and *b2* (β minor). Mouse embryonic hemoglobins are first expressed at 9.5 days of gestation in the yolk sac and later in fetal liver. The switching from predominantly embryonic to predominantly adult hemoglobin is completed between days 14 and 15 of gestation in the normal fetus. The *b1* and *b2* genes are then expressed in fetal liver and spleen and, finally, in bone marrow furing adult life. The *b1* gene is reported to be responsible for ~80% and *b2* for ~20% of adult β-globin production.

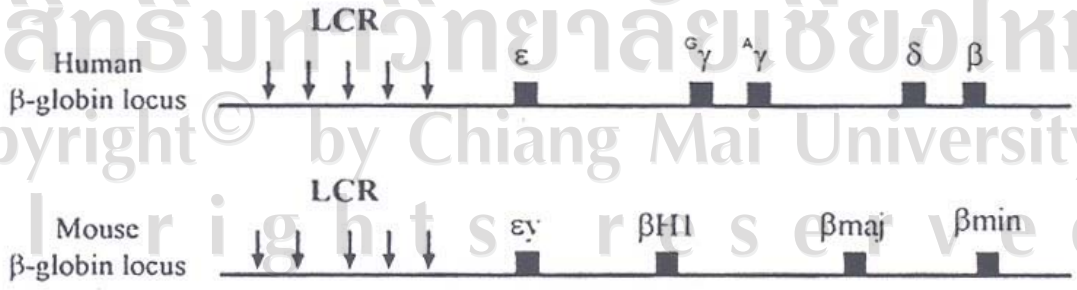

Figure 1-2 Human and mouse β-like globin loci.

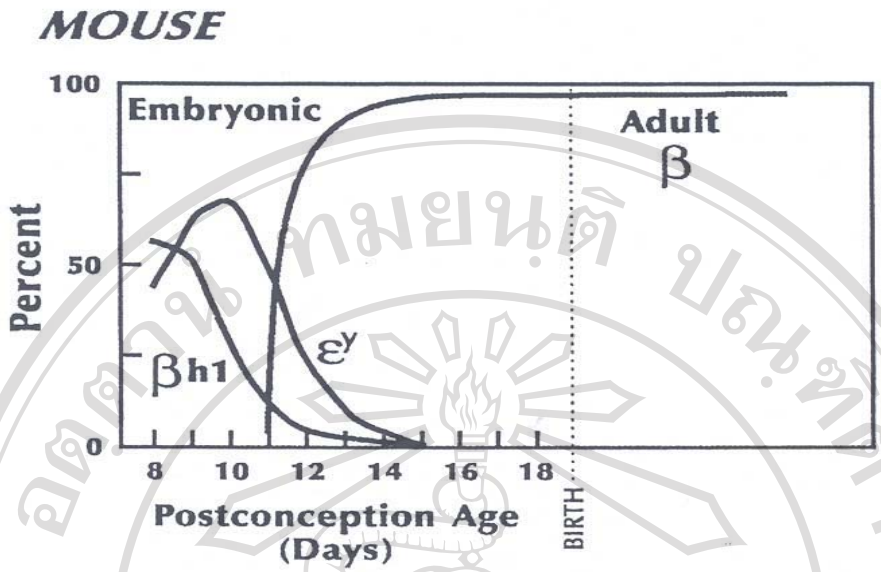

Figure 1-3 Hemoglobin switching in mice (Benz, 1980; Huo et al., 2009).

The murine ε^{y} gene is homologous to human ε. The β*h1* gene is homologous to γ-globin gene while the murine β *minor* and β *major* are homologous to human β-globin gene.

1.6 Liver fibrogenesis

Hepatic stellate cells (HSCs) also referred as Ito cells, vitamin A-storing cells, fat-storing cells or lipocyte, are found in the space of Disse, which located between parenchymal cell plates and sinusoidal endothelial lining cells of the hepatic lobule. They store 80% of vitamin A (retinoid) in the whole body in the form of lipid droplets in their cytoplasmic processes, in physiological condition, quiescent HSCs play pivotal role in the regulation of retinoid homeostasis. In chronic injury, HSCs display proliferation and fibrogenic myofibroblast phenotype. This transformation process, termed 'activated HSCs' that lack both lipid droplets and long processes. They also express of alpha-smooth muscle actin (α-SMA) and synthesis a largement of extracellular matrix (ECM) component especially collagen. Increased synthesis of collagen leads to fibrosis and eventually cirrhosis.

The clarification of stellate cell responses in hepatic injury and repair has been a significant turning point in understanding the basis of hepatic fibrosis. In particular, the identification of stellate cell activation as a key event in fibrogenesis has provided

an important frame work for conceptualizing the liver's response to injury. HSCs activation refers to the conversion of a resting vitamin A-rich cell to one that is proliferating, fibrogenic and contractile. Moreover, HSC activation represents a continuum, such that early changes in cellular phenotype may be distinct from those occurring with progressive injury and activation in terms of growth characteristics, response to soluble mediators, inflammatory signaling, and apoptotic potential. A number of elegant studies have characterized the cellular and molecular mechanisms. Cellular changes accompanying HSC activation include increasing and enlargement of rough endoplasmic reticulum, ruffled nuclear membrane, and appearance of contractile filaments. The initiation phase is due primarily to paracrine stimuli from injured neighboring cells, which include hepatocytes, Kupffer cells, sinusoidal endothelial cells, platelets, and infiltrating inflammatory cells. Some of these cells (i.e. hepatocytes, Kupffer cells) promote HSC activation by producing lipid peroxides leading to oxidative stress. A number of cytokines released by damaged neighboring cells also can activate HSC.

The interaction of HSCs with the ECM is also of major importance in the profibrogenic behavior of activated HSCs. Following chronic injury, HSCs activate or transdifferentiate into myofibroblast-like cells, acquiring contractile, proinflammatory and fibrogenic properties. They secrete fibrillar collagens and other ECM proteins. Increased collagen mRNA stability mediates the increased collagen synthesis in activated HSCs. Interestingly, quiescent HSCs express markers that are characteristic of adipocytes (PPARγ, SREBP-1c, and leptin), while activated HSCs express myogenic markers (α-SMA, c-myb and myocyte enhancer factor-2).

The altered microenvironment can amplify the fibrogenic activity of HSC by different mechanism. First, fibillar collagens can bind to discoidin domain receptor (DDR), recently described in HSC, and activate intracellular signaling pathways. Second, the altered ECM can serve as a reservoir for a number of growth factors (PDGF, TGF-β, FGF) and MMPs. PDGF, mainly produce by Kupffer cells, is the predominant mitogen for activated HSC and it is also produced in part by HSC themselves. Conversely, TGF-β is mainly autocrine. Third, HSC express a number of integrins, heterodimeric transmembrane proteins whose ligands are matrix molecules rather than cytokines, which transduce the extracellular signals from the ECM in to

the cells. Activated HSC migrate in response to cytokines released by monocytes and secrete a number of proinflammatory cytokines and chemokines that could participate in the activation of lymphocytes and the recruitment of white blood cells, thus amplifying the inflammatory response. Fibrosis is influenced by different T-helper subsets, the Th2 response being associated with more active fibrogenesis. Kupffer cell play a major role in liver inflammation by releasing ROS and cytokines. Damaged hepatocytes release ROS and fibrogenic mediators and induce the recruitment of white blood cell by inflammatory cell. Apoptosis of damaged hepatocytes stimulates the fibrogenic actions of liver myofibroblasts. A vicious circle in which inflammatory and fibrogenic cell stimulate each other is likely to occur.

Table 1-1 Factors cause liver fibrosis in animals (modified from (Wallace et al., 2008)).

Causing agents	Acute mechanisms of damage	Histological evidences
Carbon tetrachloride	Lipid peroxidation	Centrilobular hepatocyte death
Thioacetamide	Reactive thioacetamide sulfoxide causes non-specific covalent binding of cell constituents	Centrilobular hepatocyte death
Dimethylnitrosamine	CYP-metabolized reactive formaldehyde methylates cell constituents and causes apoptosis	Hemorrhagic centrilobular necrosis, destruction of sinusoidal endothelial cells leads to coagulation
Pig serum	Portal vein thickening, haemodynamic effects, cell damage/apoptosis	Not overt damage to hepatocytes (no increase in ALT, ALP)
Bile duct ligation	Raised liver bile acids stimulate hepatocyte apoptosis and necrosis (cell membrane disruption)	Periportal hepatocyte death
Methionine- and choline-deficient diet	Lipid accumulates in hepatocyte. Oxidative stress results in hepatocyte necrosis and inflammation	Lipid accumulation occurs in the centrilobular regions of the liver and in the portal tract region
Alcohol	Increased gut permeability to endotoxin resulting in hepatic exposure to ROS and inflammation	Lipid accumulation occurs in the centrilobular regions of the liver. Inflammation and necrosis in the centrilobular region
Concanavalin A	Hepatocyte necrosis observed only after initial treatments. Inflammatory infiltrate persists, particularly centrilobular region	Centrilobular and perisinusoidal fibrosis
Excessive iron	ROS-induced lipid peroxidation	Periportal hepatocyte apoptosis

Table 1-2 Common tests and biomarkers for liver fibrosis (Wallace et al., 2008).

Marker	Comments
Non-invasive tests	
Diffusion weight MRI	Measures the apparent diffusion coefficient (ADC) of water. Assessed for the diagnosis of liver fibrosis in patients with chronic HCV [224]
Elastography (Fibroscan)	Ultrasound (5 MHz) and low-frequency (50 Hz) elastic wave detection: propagation velocity through tissues is related to elasticity [225]
Tests requiring blood analysis	
APRI	Aspartate aminotransferase to platelets ratio index [226]
ELF	Serum hyaluronic acid, N-terminal propeptide of type III collagen and tissue inhibitor of matrix metalloproteinase 1 plus age [227]
Fibrometer	Hyaluronate, prothrombin time, platelets, AST, α_2-macroglobulin, urea and age [228]
FIBROSpect II	Hyaluronic acid, tissue inhibitor of metalloproteinases 1 and α_2-macroglobulin [229]
FibroTest	FibroTest is used for the assessment of fibrosis, ActiTest for the assessment of liver necrosis [230]. The test determines α_2-macroglobulin, haptoglobin, GGT, total bilirubin, apolipoprotein A1 and alanine aminotransferase
Forns test	Combining age, GGT, cholesterol and platelet count [231]
Hepascore	Bilirubin, GGT, hyaluronic acid, α_2-macroglobulin, age and sex [232]
MP3	Procollagen type III N-terminal peptide (PIIINP) and matrix metalloproteinases (MMP)-1 [233]
Test requiring liver tissue*	
Parameter associated with pro-fibrogenic cell numbers	
α-Smooth muscle actin	Primarily via immunohistochemical staining. Detects myofibroblasts (see Figure 5). Smooth muscle cells within blood vessels are also positive, therefore interpretation is required to assess the degree of fibrosis. Western blotting liver extracts will not distinguish myofibroblast-associated α-smooth muscle actin
Vimentin	Primarily via immunohistochemical staining. Detects fibroblasts associated with portal tract fibrosis (see Figure 5)
Direct determination of fibrosis	
Sirius Red stain	Relatively simple histochemical stain primarily for collagen type I (see Figure 5). Vessels are positive in normal liver, therefore interpretation is required to assess the degree of fibrosis. Also Masson's trichrome stain [238]
Collagen 1A1	Immunohistochemical staining [239]. Vessels are positive in normal liver, therefore interpretation is required to assess the degree of fibrosis
Hydroxyproline	Collagens have a high proportion of hydroxyproline residues. Biochemical assay [240] to detect total liver hydroxyproline but high control tissue values result in small overall fold changes in fibrotic liver
Reticulin	Stains collagen type III, which is highly glycosylated and therefore visible through silver staining techniques [241]
Shikata's orcein	Detects elastic fibres: this is a marker of long-standing fibrosis

1.7 Management of liver fibrosis underling fatty liver and iron overload

Iron chelation therapy

Iron chelation therapy is one of medical practices for thalassemia patients with iron overload that can improve quality of life (QOL) and prolong survival of the patients (Kushner et al., 2001). Ideally, an iron-chelating drug should be economic, orally effective, selective for ferric ion, soluble in both water and lipids, affordable and reasonably non-toxic. Hitherto, leading chelators are available commercially for which there is sufficient clinical experience in thousands of patients to allow reasonably balanced decisions regarding their suitability (Hershko, 2006).

1) Desferrioxamine (DFO, Desferol®) is produced from *Streptomyces pilosus*. It forms a 1:1 hexacoordinate octahedral complex with ferric ion, ferroxamine (FO). The drug has been the most widely treatment of choice for transfusional iron overload. The molecule is relatively large and hydrophilic, and renders orally ineffective (Porter, 2001). Adverse effects and cumbersome in subcutaneous infusion of DFO are a serious limiting factor in treatment (Wong and Richardson, 2003). DFO is expensive, so most of poor thalassemia patients cannot afford this treatment.

2) Deferiprone (1,2-dimethy-3-hydroxypyrid-4-one, DFP, L1, Ferriprox®, Kelfer®) is a synthetic oral bidentate chelator (Hider et al., 1982). It has been used over 20 years for treatment of β-thalassemia with iron overload to ensure a negative iron balance (Barman Balfour and Foster, 1999; Hoffbrand et al., 2003). DFP is rapidly absorbed, quickly reaches peak concentration in plasma compartment, and eliminated principally by liver glucuronidation (Brittenham, 2003). Apparent adverse effects of the DFP treatment include nausea, vomiting, GI disturbance, leukocytopenia, thrombocytopenia and arthopathy (Olivieri et al., 1998). For well-designed prospective clinical trial, progression of hepatic fibrosis has not been found (Wanless et al., 2002).

3) Deferasirox (*bis*-hydroxyphenyl-triazole, DFX, ICL670, Exjade®), an oral tridentate chelators, is marketed by Novartis Pharma. Its half-life of 8-16 hours allows once daily administration of the drug slurry. Iron excretion is mainly in the feces. Dose-related increases in iron excretion and absence of significant acute side effects other than gastrointestinal disturbances and a diffuse rash are increasingly used for

treatment of iron overload (Nisbet-Brown et al., 2003). However, acute side effects of this chelator mirrored those found in earlier studies (Cappellini et al., 2006).

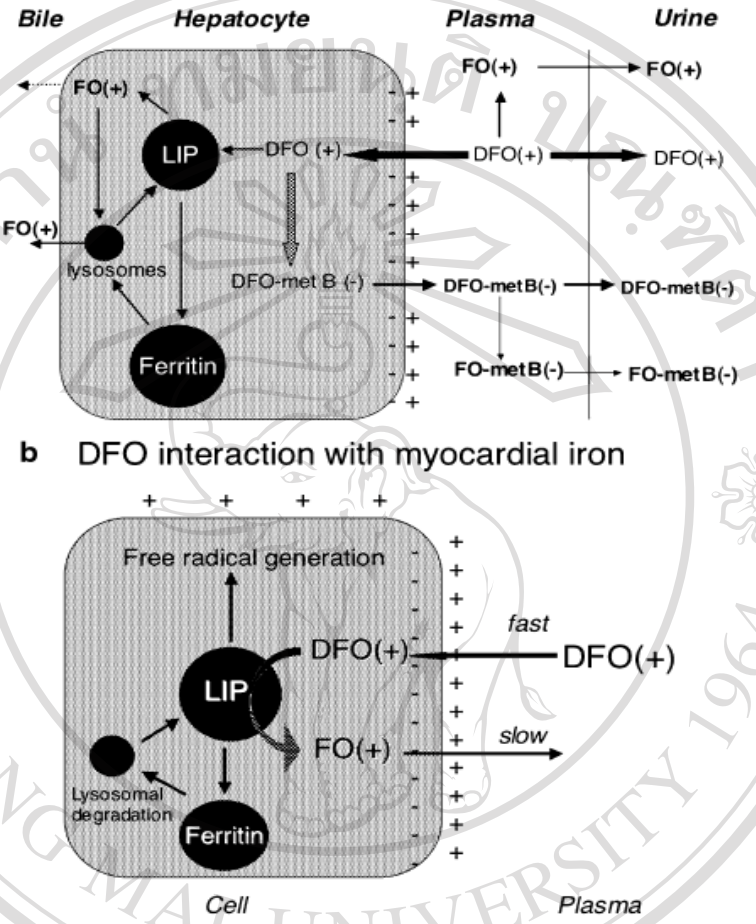

Figure 1-4 Interaction of DFO with hepatocyte and myocardial iron pools (Porter et al., 2005).

Porter and colleagues (Porter et al., 2005) have showed that hepatocellular iron consists of cytosolic labile iron (LIP) which is chelatable rapidly, cytosolic and lysosomal catabolized ferritin irons which are hardly chelatable. Positively charged DFO enters hepatocytes though facilitated uptake. If LIP is large, most of the DFO will bind iron and DFO will not be metabolized. The FO formed is initially cytosolic, but later congregates in lysosomes before leaving this compartment and entering a precanalicular compartment and ultimately biliary excretion. If LIP is low, then DFO

is metabolized to the negatively charged metabolite B before it can bind iron and to other neutrally charged minor metabolites.

1.8 Adjuvant therapy

Green tea catechins

Interestingly, several phytochemicals including bioflavonoids and tea polyphenols found in green tea and black tea exhibit antioxidant properties. In vitro studies have shown that tea polyphenols protects RBC membrane and cytosol from lipid peroxidation (Biswas et al., 2005; Dai et al., 2006; Halder and Bhaduri, 1998; Zhang et al., 1997). Pathophysiology of thalassemia relates to membrane damage, antioxidants therefore seem to be useful in protecting the RBC, prolonging RBC life span and ameliorating anemia in transfusion-dependent patients. A clinical trial of tea is currently underway, green tea catechins may prove to be adjunctive treatment for thalassemia as well as hemoglobinopathies (Rund and Rachmilewitz, 2000).

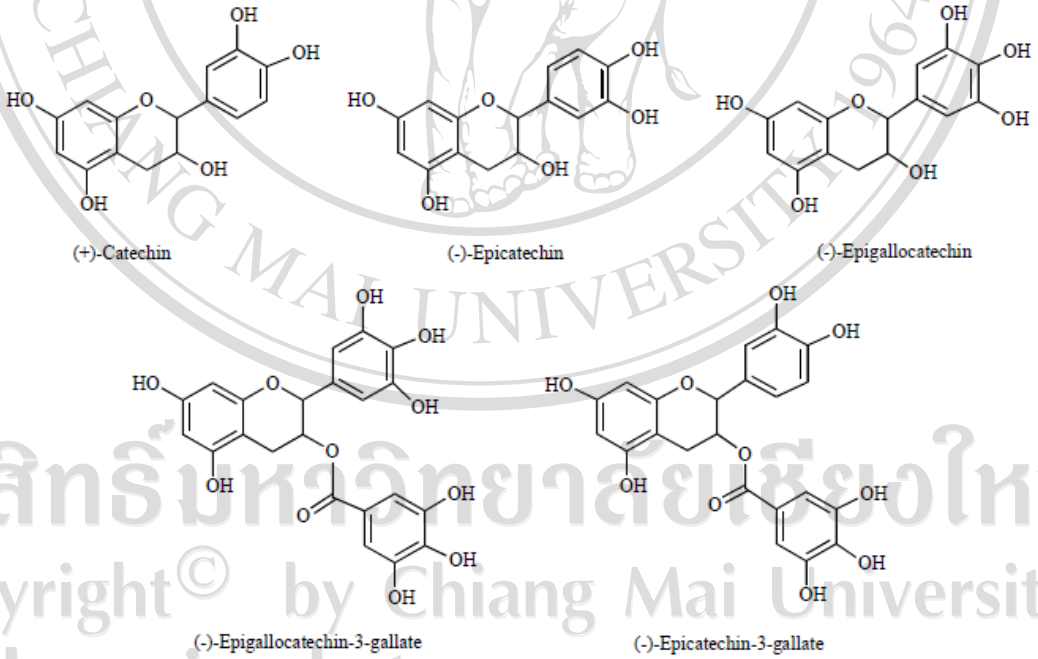

Figure 1-5 Chemical structures of catechins (Srichairatanakool et al., 2006).

Demand for green tea has increased due to human health concerns and preference. Main components in green tea are polysaccharides, flavonoids, vitamins, γ-aminobutyric acid (GABA), catechins and fluoride. Much than others, green tea

catechins (GTCs) have been of focus for the strong antioxidant capacity. Pharmaceutical activities of the components have been studied (Crespy and Williamson, 2004; Higdon and Frei, 2003; Zaveri, 2006). Five major catechins include catechin (C), epicatechin (EC), epicatechin 3-gallate (ECG), epigallocatechin (EGC) and epigallocatechin 3-gallate (EGCG), which EGCG is the most abundant and exerts strongest antioxidant capacity (Anghileri and Thouvenot, 2000). Importantly, catechins possess free radical scavenging and iron chelating properties (Ounjaijean, 2004; Ryan and Hynes, 2007). Such properties of GTCs from *in vitro* and *in vivo* studies support their therapeutic potential in reduction of oxidative stress (Ounjaijean, 2004; Ounjaijean et al., 2008; Srichairatanakool et al., 2006; Thephinlap et al., 2007).

Chitosan

Chitin, (*N*-acetyl-D-glucosamine)$_n$, is a major component of shell of crustaceans, exoskeleton of insect, and cell wall of fungi and certain yeasts. It can be deacetylated chemically and enzymatically to form chitosan that contains a certain amino groups in the molecule. Chitosan can be depolymerized by chemical hydrolysis (e.g. HCl, H_2O_2, HNO_2), enzyme hydrolysis (e.g. papain, chitosanse, lysozyme, cellulase, lipase, pectinase) and radiation (Muzzarelli et al., 1994). The polymeric backbone of chitosan (α- or β-forms) is highly polar and capable of forming hydrogen bonds with adjacent chains. Importantly, polycationic chitosan molecule potentially binds electrostatically bind negatively charged molecules such as silica (forming aerogel). Chitosan is one of commercial biopolymers that its stability, chemical properties and biocompatibility are useful for many potential applications including pharmacological, biomedical, agricultural, food, and waste treatmet protducts (Hirano S, 1990). Biomedical uses of the chitosan include packaging films, periodontal use, drug delivery, anti-oxidant, anti-microbial proterty, weight-lowering and hypocholesterolemic properties, hemostatic hydrogel and wound healing.

Chitosan is a primary ingredient in dietary weight-loss supplements. Its activity could be binding and trapping of dietary fat, leading to fat excretion and weight loss without caloric restriction. Previous studies showed that chitosan had fat-binding properties in vitro and increased fecal fat excretion in rats and mice when they are fed high amounts (3% to 15% wt/wt of the diet). Chitosan caused clinically

significant fat malabsorption and subsequently led to weight loss in human dosing 6.3 g/day (around 100 mg/kg/day) in one experiment and 4x250 mg/day (around 17 mg/kg/day) in another experiment. Importantly, fecal fat excretion is a more direct measure of the effects of chitosan because any fat not absorbed must be excreted. Controversially, some studies reported that chitosan did not affect weigh loss and fecal fat excretion. When ingested, chitosan develops an HCl-layer in the stomach. As capsulated particles of chitosan move into the duodenum, the HCl-layer becomes diluted and the chitosan particles form agglomerates with fatty acids and cholesterol, thus reducing lipid absorption from the gastrointestinal tract. Chitosan can increase the amount of fat eliminated in feces, leading to potential use of chitosan as a dietary supplement for weight loss or serum cholesterol reduction. A double-blind, randomized clinical study has shown insignificant differences in body mass index (BMI), serum cholesterol or triglycerides in subjects receiving chitosan for four weeks, compared to those receiving placebo. It is claimed that combining chitosan with fats in a beaker (in vitro) and in intestinal lumen (in physiological condition) can lead to clumping of fat and malabsorption of dietary fat respectively. The chitosans are also examined for their weight-losing and hypolipidemic effects in pre-fatten as well as fattening rats. Change of plasma lipid profiles and body weight, liver function and kidney function are monitored in the treated rats.

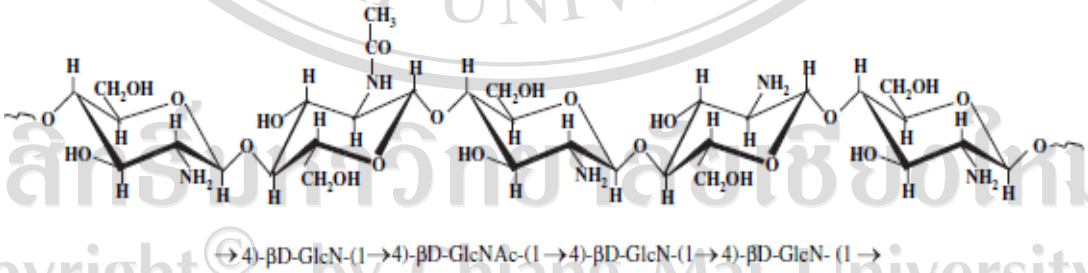

Figure 1-6 Chemical structures of chitosan.

1.9 Purposes of the study

1. To measure biochemical changes of iron and reactive oxygen intermediates (ROI) in iron-loaded cultured primary hepatocytes
2. To study efficacy of green tea catechins in alleviating iron overload and oxidative stress of the hepatocytes
3. To investigate effectiveness of chitosan in lowering nonalcoholic steatohepatitis (NASH) and liver fibrosis of high fat-fed rats