CHAPTER I

INTRODUCTION

1.1 Statement and significance of the problem

Patients with end-stage renal diseases (ESRD) who are treated with regular hemodialysis demonstrate variability of iron levels including iron deficiency, normal iron level and iron overload (Zupan, *et al* 2001). These patients are commonly found with anemia caused by erythropoietin (EPO) deficiency as well as abnormal iron absorption from gastrointestinal (GI) tract and loss of folic acid during hemodialysis. In general, EPO, a peptide hormone produced by kidneys, increases hemoglobin production and help to correct the anemia due to the iron and folic acid loss. Due to insufficiency or lack of produced EPO, it is therefore an essential therapeutic hormone for anemia in these patients in combination with oral or parenteral iron (Wingard, *et al* 1995). However, EPO treatment is quite very expensive in Thailand and may not be afforded by these patients. Intermittent blood transfusion is an alternative therapy to maintain their blood hemoglobin level above 10 g/dl.

Iron overload in ESRD patients is caused by erythropoietin hyporesponsiveness and multiple blood transfusions (Tamg, *et al* 1999). Non-transferrin bound iron (NTBI) is a potentially toxic form of iron present in such many iron-overloaded patients as β-thalassemia, hereditary hemochromatosis and post-bone marrow transplantation (Ahmed, *et al* 1986, de Valk, *et al* 2000, Durken, *et al* 1997). It usually appears in fully or highly saturated plasma transferrin; as a result of multiple blood transfusion, increased iron absorption or hemopoietic suppression. NTBI is a chemical catalyst required for the formation of reactive oxygen species (ROS) including hydrogen peroxide, superoxide anion and hydroxyl radical in the Haber-Weiss and Fenton reactions. These ROS are toxic for human being and can change the structure of carbohydrates, lipids, proteins and nucleic acids. Consequently, it may damage cells, tissues and vital organs such as blood vessels, heart and liver. This leads to multiple organ dysfunction and contributes a cause of death in the patients (Bergamini, *et al* 2004).

Deferiprone (1,2-dimethyl-3-hydroxypyrid-4-one, DFP or L1) is a synthetic oral iron chelator used clinically, mainly in β-thalassemia patients (Pippard and Weatherall 2000). DFP belongs to the family of alpha-ketohydroxypyridines, a relatively new class of chelating agents, some of which are naturally occurring. It has a high affinity for binding and removing the iron bound to transporting and storing proteins in the body, and largely sparing other biologically important metals. This compound is a

moderately hydrophobic small molecule (MW 199 Da) it is quite stable in the human digestive tract and readily absorbed. DFP has undergone extensive trials in hundreds of patients all over the world and has been proved to be orally effective in removing excess iron from various parts of the body of iron-loaded patients, including the liver and particularly the heart (Diav-Citrin and Koren 1997).

Curcumin is a main compound of the phytochemical curcuminoids from *Curcuma longa* L. and available in a yellow powder with bitter taste. It is not well water-soluble or absorbed in the GI tract (Ammon and Wahl 1991). It can chemically interact with borate to form a red-colored complex (Sui, *et al* 1993), due to the transformation of keto-enol structure. Native form of curcumin can be changed into cyclic form surrounding some transition metal such as Fe^{2+}, Cu^{2+} and Ni^{2+}(Sorenson 2002). It can bind directly to free iron as well. Curcumin has many important biological and pharmacological activities. It inhibits lipid oxidation and enzymes in inflammatory process. It has been studied as anti-mutagenic and anti-cancer agents (Miquel, *et al* 2002). Patro and coworkers demonstrated that 1,3-diketones of phenol ring of curcumin can inhibit lipid oxidation better than vitamin C and E. Hydroxyl group of phenol ring is related to the iron-binding capacity (Patro, *et al* 2002). Therefore, curcumin with free-radical scavenging and iron-chelating activity inhibits and prevents cell and tissue injuries caused by free radicals.

The ESRD patients on regular hemodialysis, whom are treated with intermittent blood transfusion or regular EPO injection together with iron supplement, may meet the oxidative stress and iron overload (Eschbach and Adamson 1999). The surface contact of red blood cells on dialysis membranes as well as increased iron intake by blood transfusion or supplement iron may produce a large number of free-radicals which potentially damage tissues and vital organ as previously mentioned.

Curcumin could be a potential therapeutic or preventive phytochemical for patients with increased free radicals and iron overload. Possibly, it can bind to the harmful chelatable plasma iron as NTBI and also reduce the fragility of red blood cells and the oxidative damage of other tissues caused by free radicals. Based on its biological properties, curcumin may potentially be synergistic or improve the treatment with deferiprone in the ESRD patients who are treated with regular hemodialysis. Curcumin may reduce the used of deferiprone and its side effects. Therefore, future investigations of curcumin use with or without the standard iron chelator to remove NTBI are warranted.

1.2 Literature review

1.2.1 Functions of the kidney

The kidneys are paired organ system located in the peritoneal space. The functions of the kidney may be characterized as excretory, regulatory and endocrine. The excretory function serves to get rid of the body's most undesirable end products of metabolism and any excess of inorganic substances in ingested diet. Waste products include the non-protein nitrogenous compounds such as urea, creatinine and uric acid are largely excreted by the kidneys; while a number of other organic acids and amino acids are excreted in small quantities. The regulatory function of the kidney plays a major role in homeostasis (Hill 1990, Wright 1995). The endocrine functions of the kidney may be regarded primarily because the kidney is a site where some hormones are firstly produced and elsewhere activated. In its primary endocrine function, the kidney produces renin, prostaglandins and erythropoietin. Erythropoietin acts on the bone marrow to stimulate the proliferation and maturation of erythrocyte precursors (Figure1.1).

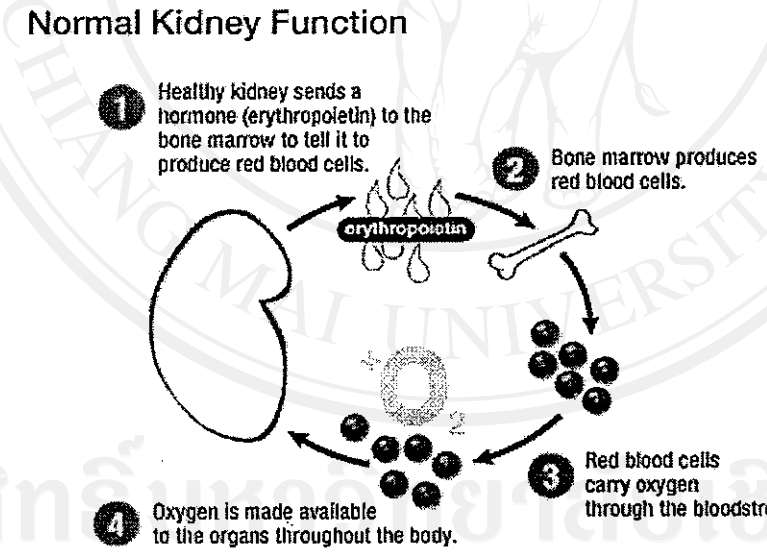


Figure 1.1 Normal kidney function (From www.aranesp.com).

1.2.2 Renal dialysis

The development of techniques for hemodialysis and peritoneal dialysis has changed end-stage renal disease from illness that once terminated fatally within a few months to a chronic illness with an average survival time of 10-15 years. Hemodialysis refers to a process in which blood solutes are allowed to diffuse down the concentration gradients, across a semi-permeable membrane and into a recipient fluid called the dialysate. The artificial kidney is an application of extracorporeal dialysis, since an extracorporeal circulation is set up via an implanted arteriovenous cannula to lead the patient's blood to the membrane cartridge immersed in the dialyzer bath (Figure 1.2). Peritoneal dialysis is intracorporeal, which the patient's abdominal cavity is perfused with (or filled with and then emptied of) the recipient fluid and dialysis occurs across the peritoneal membrane (Burrows 1995). Hemodialysis is generally more efficient than peritoneal dialysis in the removal of waste products in uremic patients, although peritoneal dialysis is of value in situations in which arteriovenous fistulas fail.

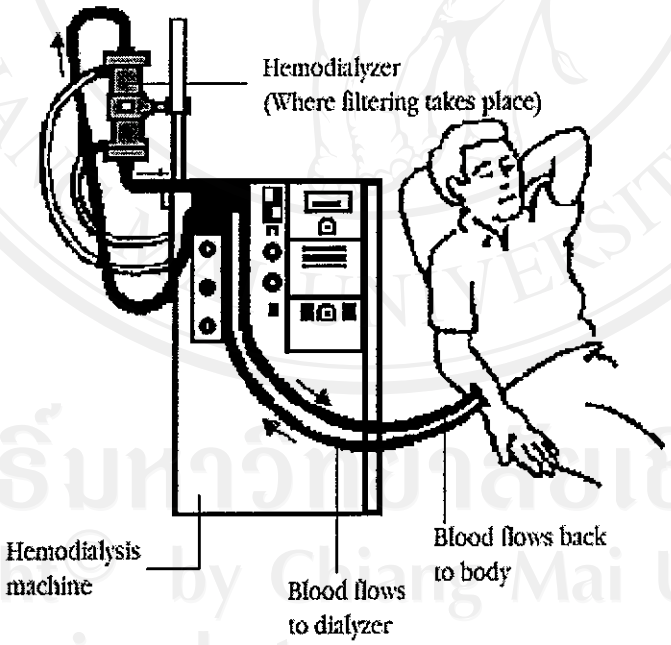


Figure 1.2 Hemodialysis procedure (From www.nkfg.org)

1.2.3 The relevance of iron in biology

Iron (Fe: element 26 in the periodic table) is the second most abundant metal (after aluminium) and the fourth most abundant element in the Earth's crust. In aqueous solution, it has two oxidation state, ferrous ion (Fe^{2+}) and ferric ion (Fe^{3+}), which can participate in many biochemical reactions. These include the electron-transport chain, the activation of molecular oxygen, nitrogen and hydrogen, the composition of noxious derivatives of oxygen such as peroxide and superoxide, the synthesis of DNA, and the binding of oxygen by hemoglobin, myoglobin and hemerythrins (Lieu, *et al* 2001). At neutral pH values, ionic composition and oxygen tension of most physiologic fluids, the stable state of iron is Fe (III). Fe (II) is readily oxidized to Fe (III) by oxygen molecules and easily identified each time, a rusty precipitate forms in a beaker of ferrous salt standing in air. The hydrolytic propensity of ferric iron (Fe^{3+}) is so great that equilibrium concentration of the free, hydrated form cannot be above 10^{-17} M in the neutral solution. Thus, living organisms have to evolve specific iron-sequestering molecules to maintain the element in solution, available for transport and biosynthesis of essential iron proteins and enzymes (Howard 1999). To inhibit the iron-catalyzed redox reactions, some iron-binding compound may lower the exposure of cells to potentially damaging reactive oxygen species (ROS) such as H_2O_2, $O_2^{\bullet-}$ and $OH^{\bullet}$.

Iron is essential for the growth of almost all living organisms and involved in a large number of biological systems. These are oxygen transport, activation and detoxification, nitrogen fixation and many reactions of photosynthesis. When iron is present in excess or in forms where the electrons are incompletely coordinated, it has potential to participate in the generation of harmful free radicals, due to its facile ability to redox cycle between the Fe^{2+} and Fe^{3+} oxidation states.

1.2.4 Human iron metabolism

The average iron content of a healthy human is 40 -50 mg per kg body weight, the majority of which is present as hemoglobin (30 mg/kg body weight). About 5 mg/kg body weight is present in myoglobin in muscle tissues and 2 mg/kg body weight is present in cells as iron containing enzymes. The remaining body iron is present in the storage form of ferritin or hemosiderin, predominantly in liver (17 mg/kg body weight), spleen and bone marrow. Storage iron is usually between 0 and 2000 mg, depending on the balance between available dietary iron and the iron requirements of the individual (Crichton and Charloteaux-Wauters 1987). In healthy humans iron-containing molecules can be divided into storage iron pools, transit iron pools, functional iron pools and regulatory iron pools (Figure1.3)(Andrews 1999).

In iron overload, additional forms of iron may be found including plasma non-transferrin bound iron (NTBI) forms.

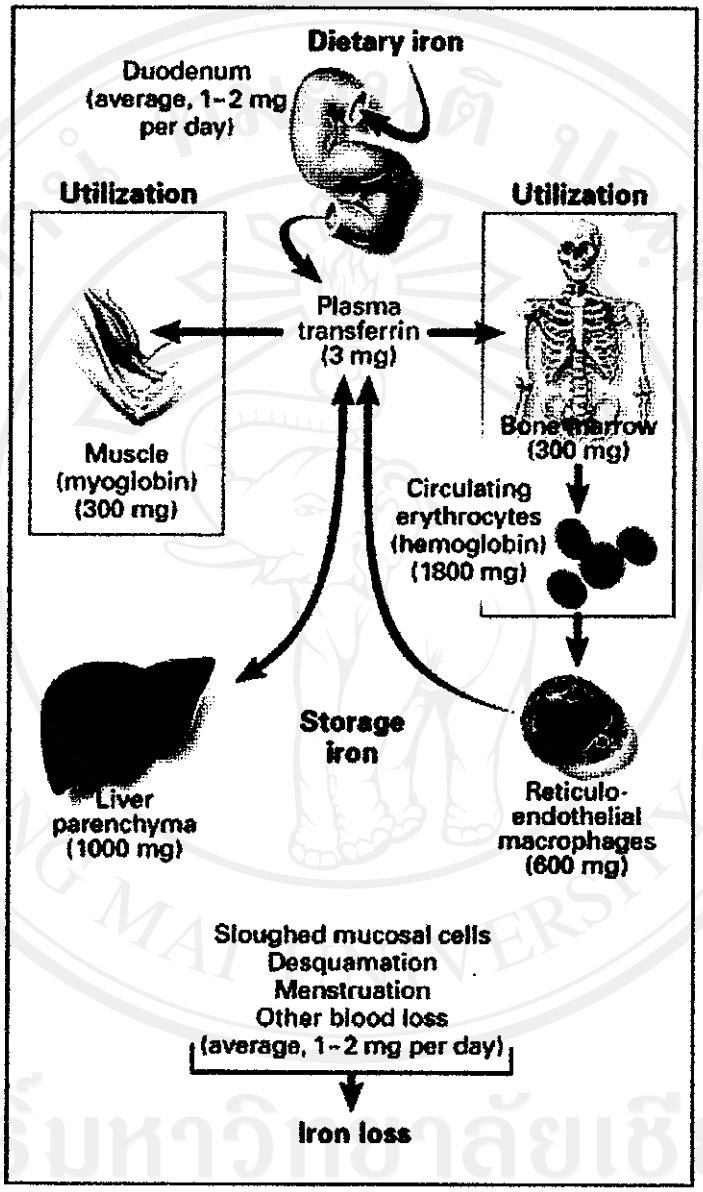


Figure 1.3 Distribution of iron in adults (From Andrew, 1999)

1.2.5 Iron absorption

Iron balance is physiologically regulated through the control of intestinal iron absorption. Absorption of dietary iron is highly conserved since no specific mechanisms for iron excretion are available except for the obligatory losses through exfoliation of gastrointestinal mucosal cells, bile, urine and skin in small proportions. Around 0.5-2 mg of iron is lost daily and balanced by controlled dietary iron absorption of 0.5-2 mg per day by upper intestinal mucosa. Most of the dietary iron is absorbed in the duodenum and the jejunum, mainly as Fe (II) and only to a small extent as Fe (III) (Finch and Huebers 1986).

The regulation of iron absorption is still incompletely understood but is increased by a number of well identified factors such as iron deficiency, anemia, hypoxia and iron turnover. Iron transport through the enterocytes can be seen as two-stage process: the first involves uptake across the brush border (apical) membrane and the second is the transfer of iron across the basolateral membrane into blood stream. The duodenum appears to be the part of the intestine which can modulate its iron absorption the most. Absorption of heme iron is increased in iron-deficient patients and is relatively greater than non-heme iron absorption (e.g. four-fold at serum ferritin of 100 μg/l or approximately two-fold at serum ferritin of 30 μg/l). Increased plasma iron turnover is associated with increased iron absorption, but iron storage is inversely related to iron absorption. It is known that iron absorption across the brush border epithelium of small intestine is independent of the transferrin receptors (TfR) which has been localized to the basolateral but not the luminal surface of enterocytes (Bezkorovainy 1989). However, iron is likely to be reduced to the ferrous state before absorption since there is evidence of ferrireductase activity at the microvillus border (Figure 1.4).

The product of the *HFE* gene has been shown to be important for iron absorption. Over 80% of adult hemochromatosis patients are homozygous for a missense mutation (Cys282Tyr or C282Y) in the *HFE* gene, initially termed HLA-H. The second mutation (His63Asp or H63D) has been found in a significant number of compound heterozygote with clinical features of genetic hemochromatosis (Thielen, *et al* 2004). Structural analysis of the *HFE* protein has shown a completely non-functional peptide-binding site including folding of a_1 domain and inaccessible tyrosine residues for peptide-binding (Feder, *et al* 1996). Due to the C282Y mutation, the disulfide linkage in this region is broken, preventing the association between *HFE* and β_2-microglobulin that is necessary for further processing and transport to the cell membrane. Hence, *HFE* is likely to have a functional role on the cell surface but does not appear to bind peptide.

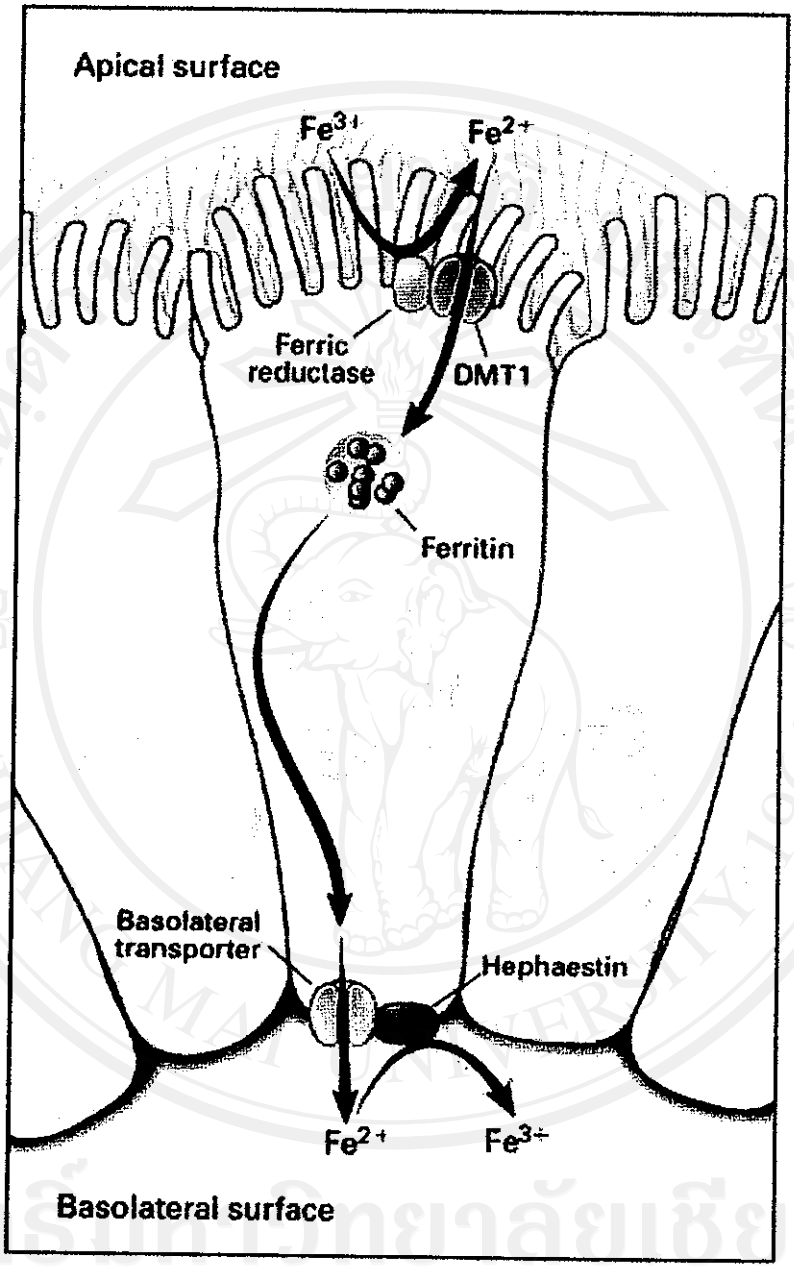


Figure 1.4 Iron transport across the intestinal epithelium (From Andrew, 1999)

HFE protein is found in the sinusoidal epithelium and Kupffer cells, and on the basolateral surface of the intestinal mucosal cells. Laboratory investigation has shown the interaction between *HFE* and TfR on the plasma membrane (Gross, *et al* 1998), the quaternary complex formation of *HFE* with TfR and transferrin (Lebron, *et al* 1998) and the effect of *HFE* to reduce transferrin-mediated iron uptake in HeLa cells (Roy, *et al* 1999). The presence of *HFE* within the crypt cell may serve as a sensor of body iron status, either directly or by its interaction with the TfR on the basolateral surface and within endosomal compartments.

There is the important evidence for an endosomal iron transport protein, *Nramp* (natural resistance-associated macrophage protein) family, which it is also important for the intestinal iron uptake step. Mutations in this protein are responsible for the microcytic anemia phenotype in *mk* mouse (Flemming, *et al* 1997) and the Belgrade rat (Flemming, *et al* 1998, Gunshin, *et al* 1997). This metal transport protein, also referred to a divalent-cation transporter protein-1 (DCT1), is ubiquitously expressed (particularly in the proximal duodenum) to induce the uptake of ferrous ions and transports a variety of other divalent metal ions by a mechanism coupled to a proton gradient (Gunshin, *et al* 1997). DCT1 has been identified as the rat isoform of *Nramp2* because of its homology to *Nramp1* that is found only in macrophages. There is an abundance of DCT1 mRNA in the mucosa of the proximal small intestine. It is likely that *Nramp2* (DCT1) is the key protein that imports iron from the human intestinal lumen. It has been suggested that *HFE* may influence the enterocyte precursor to absorb iron appropriately by modulating *Nramp2* expression (Griffths, *et al* 1999). With respect to the transfer step, Hephaestin has recently been implicated (Vulpe, *et al* 1999) and preliminary findings with the cloning of novel transporters have been reported (Mckie 1999) which may also be involved in the transfer step. Other factors are undoubtedly involved in regulation of iron absorption from the gut. It has long been known that membrane-bound reductase of duodenal mucosa plays a key role in iron absorption by reducing ferric iron to the ferrous state (Riedel, *et al* 1995) before its active uptake.

1.2.6 Proteins involved in iron transport and storage

Transferrin

Physiologically, the majority of cells in the organism acquire iron from a well-characterized plasma glycoprotein, transferrin (MW approximately 80 kD) (Richardson and Ponka 1997) Transferrin consists of two homologous domains, each of which contains a high affinity Fe (III)-biding site. Binding affinity of iron to transferrin is a pH-dependent process. In plasma (pH approximately 7.4), transferrin binds iron very

strongly (K_d approximately 10^{-23} mol/l), whereas virtually no binding occurs at pH≤5, and this property plays an important role in the physiological mechanism of iron release transferrin (Brock 1989). Transferrin binds iron via phenolate oxygens of two tyrosine residues, an imidazole nitrogen of a histidine residue and a carboxylate oxygen of an aspartic residue (Anderson, *et al* 1989). These protein ligands occupy four of six octahedral sites around each iron atom, leaving two *cis* positions to be filled by the anion carbonate (or bicarbonate). Binding and release of iron by transferrin are accompanied by dramatic conformational changes in the protein. In the absence of iron, the two domains involved in the binding are widely separated and assume an "open" configuration. On the other hand, insertion of iron brings two domains of the binding cleft close toward the metal, and transferrin assumes "closed" conformational state (Baker and Lindley 1992).

Transferrin functions to transport iron between sites of absorption, storage and use. Although the transferrin-to-cell branch of the metabolic iron cycle is reasonably well known, the mechanism and regulation of iron mobilization and transport from tissue stores to plasma transferrin are the least understood aspects of iron metabolism. Transferrin receives most of iron from hemoglobin catabolized by macrophages of the reticuloendothelial system (for example, Kupffer cells). Senescent erythrocytes are internalized by the macrophages that liberate iron from its confinement within the protoporphyrin ring by the action of heme oxygenase and then release iron almost quantitatively to transferrin in the circulation (Finch and Huebers 1986). Unfortunately, the mechanisms and controls involved in the release of iron from macrophages and other cells have not been defined. Interestingly, studies of patients with recently identified genetic deficiency of ceruloplasmin suggest that iron may be released from cells of many tissues, and not just the liver and macrophages as once thought. Ceruloplasmin is a blue copper-containing protein with ferroxidase activity. Patients with hereditary acerulopasminemia have low plasma iron levels, but marked iron accumulation in the liver, pancreas, brain and also kidney (Mukhopadhyay, *et al* 1998). Once possible explanation of iron overload in aceruloplasminemia patients is that the release of iron from the cells requires the ferroxidasse activity of ceruloplasmin. It is conceivable that ceruloplasmin may facilitate cellular iron release by promoting the oxidation of Fe (II), the redox form in which iron appears to be within the intracellular "transit" pool (Breuer, *et al* 1995). In this regard, it may be pertinent to mention that yeast *Saccharomyces cerevisiae* posses a membrane-spanning ferroxidase (Fet3) that has homology to the multicopper oxidases, including ceruloplasmin, and that is required for high-affinity iron transport (Askwith, *et al* 1994).

Hemodialysis patients with normal or even increased iron in stores sometimes develop resistance to erythropoietin therapy (Eschbach, *et al* 2002). This condition is referred as "functional iron deficiency" and is caused by inadequate mobilization of ferritin iron during rapid hemoglobin regeneration. In severe iron overload patients, plasma can not only contain transferrin completely saturated with iron but also a chelatable low-molecular-weight iron fraction not associated with transferrin.

Transferrin receptor

Transferrin is recognized by specific cell membrane receptors that are gatekeepers responsible for physiological iron acquisition by most cell types in the organism (Richardson and Ponka 1997). The transferrin receptor consists of a disulfide-linked transmembrane glycoprotein homodimer having a molecular radius (M_r) of 180 kDa, and each subunit (90 kDa) binds one molecule of transferrin. The human transferrin receptor contains a small N-terminal cytoplasmic domain of hydrophilic character having a molecular mass of 5 kDa and frequently contains a phosphate group bound to the hydroxyl moiety of serine-24. However, the phosphorylation and dephosphorylation of this latter residue is not required for controlling endocytosis or recycling of the transferrin receptor. The cytoplasmic domain of the transferrin receptor is essential for receptor internalization, and a tetrapeptide sequence within the cytoplasmic tail of the transferrin acts as a signal for high-efficiency endocytosis. The cytoplasmic tail is linked to a C-terminal extracellular domain of 672 amino acids by a hydrophobic membrane-spanning segment 62 amino acids from the N-terminus, and this hydrophobic part of the transferrin receptor contains covalently bound fatty acid residues (palmitic acid) as a result of post-translation modification (Richardson and Ponka 1997). In nonerythroid cells, transferrin receptor numbers correlate negatively with iron levels in an ill-defined "transit iron pool", and the regulation of the receptor synthesis is post-transcriptional and involves modulation of transferrin receptor mRNA stability. However, the primary control of transferrin receptor expression in erythroid cells appears to be the transcription that probably plays an important role in maintaining high levels of receptor needed to support the hemoglobin biosynthesis (Ponka 1997).

Ferritin

Ferritin is a ubiquitous protein in which the only clearly defined function is the sequestration and storage of iron (Harrison and Arosio 1996, Richardson and Ponka 1997). Mammalian ferritin consists of a protein shell that can accommodate up to 4,500 atoms of iron in its internal cavity. The protein shell by itself has a molecular mass between 430 and 460 kDa, and is made up of 24 symmetrically related subunits of two types, a light subunit (L-subunit) of approximately 19 kDa and a heavy subunit (H-subunit) of

approximately 21 kDa. The amino acid sequence of the H- and L- subunits differ by approximately 50%, and the ferritin genes from several species including humans have been cloned and sequenced (Harrison and Arosio 1996). Different proportions of the two subunits give rise to the heterogeneity of ferritin observed in different tissues. The entry and exit of iron may occur via channels in the protein shell. Some channels are hydrophobic in nature and some are hydrophilic. Ferritin synthesis is inducible by iron requiring a mechanism in which iron recruits ferritin mRNA from an active pool. In addition, inflammatory cytokines and "oxidative stress" are involved in an iron-independent regulation of ferritin gene expression (Ponka, *et al* 1998).

1.2.7 Cellular iron acquisition from transferrin

Iron acquisition via transferrin receptor-mediated endocytosis, which is likely to be identical in all cell types, is schematically depicted in Figure 1.5 (Morgan and Oates 2002, Richardson and Ponka 1997). Firstly, transferrin attaches to its specific receptors on the cell surface by a physicochemical interaction, not requiring temperature and energy. Secondly, the transferrin-receptor complexes are internalized under a temperature- and energy-dependent process into the endocytic vesicles in the cells. Thirdly, Iron transport across the endosomal membrane likely requires a specific endosomal iron transporter called "*Nramp2*". Fourthly, iron is released from the transferrin within the endocytic vesicles by a temperature- and energy-dependent process that involves endosomal acidification (pH 4.5-5.5) (Morgan 1981). Finally, the apotransferrin which remains attached to its receptor at pH approximately 5.5 returns to the cell surface where it released from the cells, available for acquiring iron from macrophages and to a lesser extent from hepatocytes, gut and other cells. An influx of protons into endosome occurs via an adenosine triphosphate-dependent H^+ pump. Mild acid condition and transferrin-TfR binding promote more efficient release from transferrin iron in the endosome. Afterwards, iron is probably bound to an unidentified ligand and transported to intracellular sites of use and/or storage in ferritin. However, the nature of elusive intermediary iron pool and cellular iron trafficking remains enigmatic. Only erythroid cells exists a specific targeting of iron toward mitochondria for ferrochelatase-catalyzed heme production (Richardson and Ponka 1997). Recent evidence suggests that a transient mitochondria-endosome interaction in erythroid cells could be involved in iron translocation to mitochondrial ferrochelatase. Recent studies indicate that after saturation of the transferrin receptors, iron is acquired from transferrin taken up by the cells via low-affinity adsorptive pinocytosis. Some investigators propose that transferrin receptor-independent iron uptake

(possibly facilitate diffusion) occurring after saturation of receptors is far greater than the iron uptake via transferrin receptor-mediated endocytosis, very little is known about the mechanism of iron acquisition. It is not known whether this iron uptake is present in some cells of the kidney.

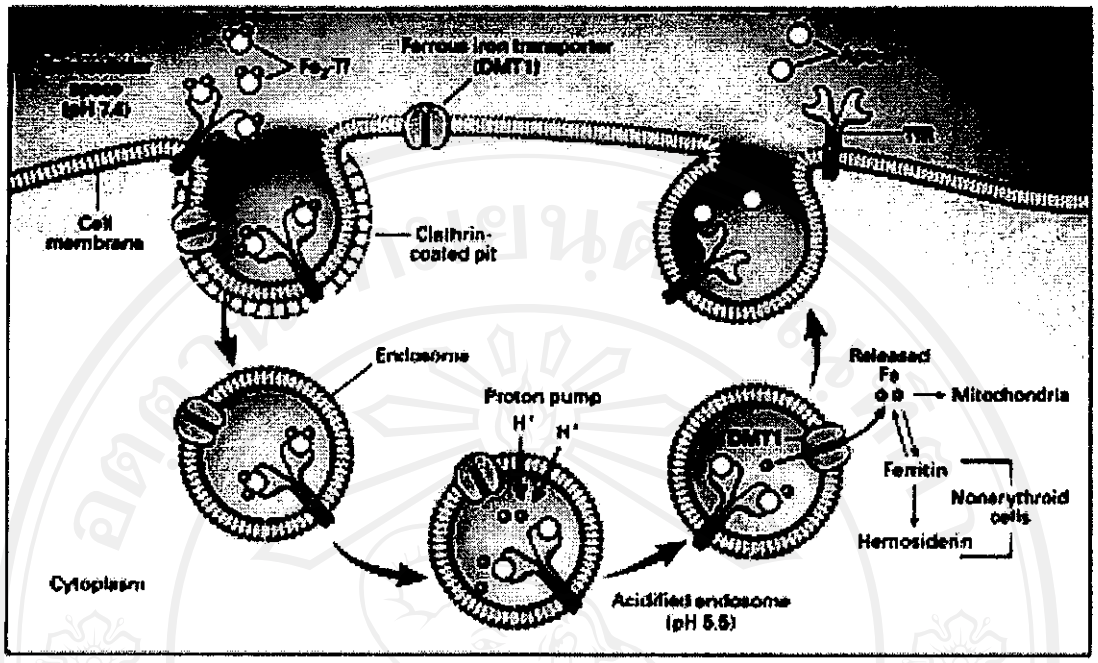


Figure 1.5 The transferrin cycle (From Andrew, 1999)

1.2.8. Regulation of iron levels in the intracellular labile pool

Labile iron pool (LIP) in cytoplasm is iron from endosome and from the breakdown of non-heme-containing proteins. It has been proposed that this LIP may be complexed to citrate, sugars, some amino acids, pyridoxal and nucleotides, but the real chemical nature of this metabolically and kinetically active pool remains elusive (Richardson and Ponka 1997). The only abiding certainty seems to be that this iron is accessible to strong chelators. Sensitive control mechanisms that monitor iron levels in the LIP and that prevent the expansion of this pool (to combat Fenton chemistry) have been developed during evolution. In general, enlargement of LIP leads to not only stimulate the ferritin synthesis but also decrease the expression of TfR. The opposite way develops when this pool is depleted of iron. Interestingly, a single genetic regulation system is responsible for these changes in expression of structurally unrelated proteins, ferritin and TfR.

In vitro research in nonerythroid cell culture demonstrated that iron-dependent regulation of both ferritin and TfR occurs post-transcriptionally and is mediated by virtually identical iron-responsive elements (IREs). IREs were first identified in the 5' untranslated regions (UTRs) of ferritin H- and L-chain mRNAs (Richardson and Ponka 1997) and were documented to mediate the inhibition of ferritin mRNA translation in iron-deprived cells. Five similar IRE motifs (contrary to single IRE in ferritin mRNAs) were later identified within 3' UTR of TfR mRNA. These IREs confer differential stability to TfR mRNA as a function of cellular iron levels. The IRE is also present in the 5' UTR of mRNA for erythroid-specific 5-amino-levulinic acid synthase, the expression of which in hemoglobin-synthesizing cells depends on the availability of iron (Ponka, *et al* 1997). The IREs are *cis*-acting nucleotide sequences, forming a stem-loop structure that contain an unpaired cytidine six base 5' of a six-membered loop, the sequence of which is CAGUGN. These hairpin structures are recognized by *trans*-acting cytosolic RNA-binding proteins known as iron-regulatory proteins (IRPs), specifically IRP-1 and IRP-2. IRP-1 shares homology with mitochondrial aconitase of the citric acid cycle. In iron-replete cells, IRP-1 contains a [4Fe-s] cluster and, posses aconitase activity and binds RNA with low affinity (Keyer and Imlay 1997). In contrast, when iron is scarce, IRP-1 binds to IREs with high affinity. The transition between the aconitase and RNA-binding form of IRP-1 occurs without changes in IRP-1 protein levels. With their homology, IRP-2 binds to IREs with similar affinity as IRP-1 and consequently represses translation of IRE-containing mRNAs. However, IRP-2 functions solely as an RNA-binding protein and regulation of IRP-2 by iron is mediated by specific proteolysis (Hentze and L.C. 1996).

The interactions of IRPs with IREs control iron metabolism in nonerythroid cells as shown in Figure 1.6 and Figure 1.7 (Becker, *et al* 1996). When cellular iron becomes limiting, the IRP-1 is recruited into high-affinity binding state. The binding of IRP-1 to the IRE in the 5' UTR of the ferritin mRNA represses the translation of ferritin, whereas an association of IRP-1 with IREs in the 3' UTR of TfR mRNA stabilizes this transcript against the ribonuclease digestion. On the other hand, the expansion of LIP inactivates IRP-1 and leads to a degradation of IRP-2, resulting in an efficient translation of ferritin mRNA and rapid degradation of TfR mRNA.

IRE/IRP system is tailored to sense the "iron-in-transit' and maintain it at appropriate levels, and iron is a player that modulates IRP-1 activity. Treatment of cell cultures with H_2O_2 causes the rapid stimulation of the RNA binding activity of IRP-1, leading to decreased ferritin synthesis (Hentze and L.C. 1996). Collectively, "oxidative stress" can dramatically affect cellular iron metabolism. Some specialized cells with specific iron/heme metabolism characteristics probably evolved additional regulatory mechanisms that can override the IRE/IRP control system. TfR expression is regulated transcriptionally in erythroid cells. In macrophages, iron increases rather than decreases TfR mRNA and protein levels, probably because of IRP-1 activation.

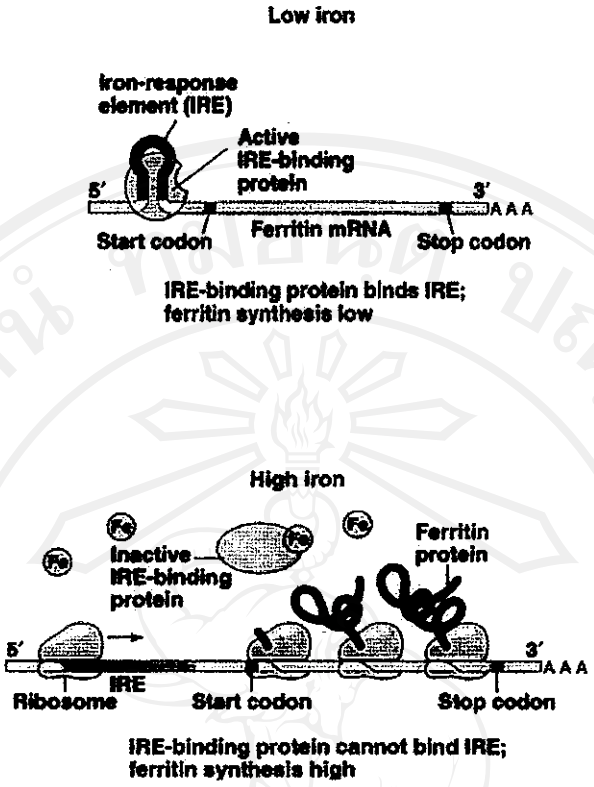


Figure 1.6 Translational control of ferritin synthesis in response to iron (From Becker et al., 1996)

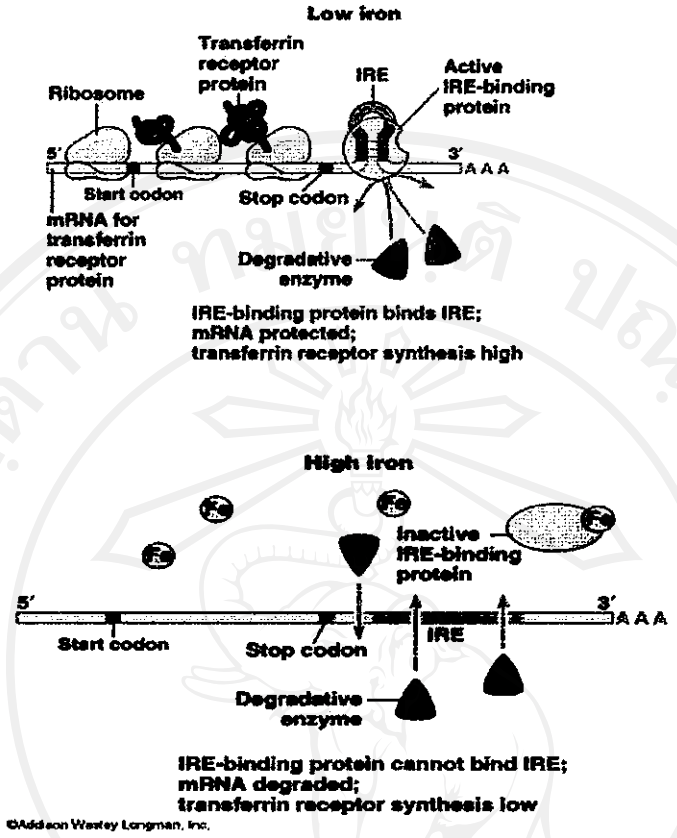


Figure 1.7 Control of degradation of transferrin receptor (TfR) mRNA in response to iron

(From Becker et al., 1996)

1.2.9 Plasma iron transport and turnover

In the aerobic extracellular environment, Fe (II) is oxidized into less soluble Fe (III) that is required for normal functions. Multicellular organisms have evolved several complexing agents including transferrin, ferritin, lactoferrin, haemopexin, haptoglobin and albumin to bind Fe (III) and maintain it in a soluble form for their utilization. Normally, the plasma iron concentration is 10-30 μM and the plasma transferrin concentration is 22-35 μM (with twice this iron binding capacity due to its two iron binding sites) so that the transferrin saturation is approximately 30%. Consequently, the plasma and intestinal fluid express unsaturated transferrin which can bind iron absorbed from the intestine or release from cells in the body. Plasma iron concentration declines during infancy and pregnancy, infections, malignancies, nephrotic syndrome, iron deficiency and after trauma. It increases in hemochromatosis, in hemolytic, hypoplastic and megaloblastic anemia. When plasma iron concentrations are elevated, plasma transferrin concentration is usually lowered and the transferrin iron-binding capacity is small. Plasma transferrin may be completely saturated in serve iron overload.

Transferrin is mostly distributed in extracellular fluid of the body, from plasma to intestinal fluid, lymph vessels and back to blood circulation. The major functions of transferrin are to maintain extracellular iron (5-7 mg of transferrin-bound iron in normal human plasma in a soluble form which is suitable for cellular uptake and to regulate iron supply to many iron-requiring cells (20-30 mg/day), particularly to developing erythroid cells, via receptor-mediated endocytosis. In addition, transferrin restricts the uncontrolled entry of excess iron into cells with the resultant risk of cellular damage. Iron requirement is 0.7-1.4 g during pregnancy and 1 mg/day during lactation (Turnbull 1978).

Plasma iron turnover rate is approximately 30-40 mg per day which mainly occurs as a result of hemoglobin catabolism from old red cells in bone marrow, liver and spleen where the sinusoids are freely permeable to transferring. Transferrin is not degraded during the process of plasma iron turnover. Some iron taken up by erythrocytes precursors in the bone marrow is not carried forward into the circulating erythrocytes but is transferred to reticuloendothelial cells when some erythroid precursors are prematurely destroyed. When circulating erythrocytes are phagocytosed by macrophages at the end of their life span, iron is finally returned to plasma transferring. A small proportion of iron goes through the liver that can exchange iron with transferrin. In iron overload, some proteins such as ferritin and albumin may play a role to scavenge an excess iron as NTBI in plasma and cleared from plasma by receptor-mediated and nonspecific endocytosis, mainly by liver and a lesser extent by other non-erythroid tissues.

1.2.10 Iron overload

Iron overload may be primarily associated with the inherited disorder of iron absorption namely hereditary hemochromatosis, or secondary to other causes such as multiple blood transfusions.

Primary iron overload

The disease described as genetic, hereditary, idiopathic or HLA-linked hemochromatosis is inherited as an autosomal recessive disorder of iron metabolism that is most commonly seen in Caucasian. Since too much dietary iron is absorbed from the gut, excess iron accumulates in many organs, eventually causing clinical manifestations such as diabetes, skin pigmentation, hypogonadism, arthritis, liver cirrhosis or cardiac failure. Iron overload may be seen in men at any age but in women is usually manifested after the age of menopause. Expression of the disorder is also influenced by dietary iron intake (Beutler, *et al* 2001).

Secondary iron overload

Secondary iron overload may be defined as a quantitative increase in total body iron that is not caused by a primary genetic defect of increased iron adsorption. Excess iron comes either from parenteral iron administration as repeated blood transfusions or from increased iron absorption from the gastrointestinal tract. Severe anemia requires regular blood transfusions, eventually suffers from iron overload and oxidative stress. When the transfused red blood cells reach the end of their lifespan and are destroyed within reticulo endothelial system, the excess iron released from catabolised hemoglobin is excreted insufficiently and finally deposited in several tissues. Transfusion related iron-loading anemia is found in β-thalassemia major, chronic renal failure, aplastic anemia, myelodyspastic syndromes and some cases of sickle cell disease (Pippard 1994).

1.2.11 Iron-catalyzed generation of free radicals

Iron is made unavailable to participate in the generation of harmful free radicals by its binding to ligands such as transferrin. In iron overload, plasma transferrin becomes saturated and NTBI is detectable. Increased quantities of low MW iron in the cells (e.g. LIP) are also potentially available to participate in free radical production. Iron is particularly important because it is present in sufficient concentration in tissues and due to the favorable redox potential of the Fe^{2+}/Fe^{3+} transition that allows easy redox cycling between Fe^{2+}/Fe^{3+}. Free radicals are defined as any species capable of independent existence that contains one or more unpaired electrons. One of the commonest reactions in the body is the iron catalysed reduction of molecular oxygen in water to sequentially form products including superoxide ($O_2^{\cdot}$), hydrogen peroxide

(H_2O_2) and the hydroxyl radical ($HO^{\bullet}$). The iron catalysed reaction between superoxide and hydrogen peroxide was first described by Haber and Weiss in 1934 and involves the sequential reduction and oxidation of Fe^{3+}(Lee and Jacobs 2004, Pippard 1999).

$$O_2^{-} + H_2O_2 \longrightarrow O_2 + OH^{-} + HO^{\bullet}$$ **Haber-Weiss reaction**

The hydroxyl radical $HO^{\bullet}$ has also found great use in studying the structural properties of DNA and DNA-protein complexes. This species can be efficiently generated via the Fenton reaction from Fe(II)-EDTA in the presence of hydrogen peroxide and a reducing agent such as ascorbate. Hydrogen peroxide is relatively stable and non-toxic by itself. Furthermore, it is an important precursor of hydroxyl radicals, requiring the availability of catalytic trace elements such as iron, copper or cobalt.

$$Fe^{2+} + O_2 \longleftrightarrow Fe3+ + O_2^{\bullet -}$$

$$2\,O_2^{\bullet -} + 2H^{+} \longleftrightarrow H_2O_2 + O_2$$ **Fenton reaction**

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

The hydroxyl radical derived from hydrogen peroxide can diffuse and abstract hydrogen atoms from DNA, resulting in cleavage of DNA molecule following subsequent chemical reaction. In the presence of iron (II) and oxygen molecules or iron (III) and hydrogen peroxide, an anti-tumor antibiotic bleomycin binds to DNA molecule and induces single-and double- strand cleavages. Upon activation, ferric peroxide abstracts hydrogen atoms from the deoxyribose rings of DNA and subsequently results in DNA cleavage. Other systems may be a metal complex, often bound to DNA (as in bleomycin) or freely diffusible species such as a hydroxyl radical.

A number of physiological scavengers of ROS such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are present in the cells and can scavenge hydrogen peroxide efficiently. Tissue damage will depend on the relative rates of formation and scavenging of harmful free radicals. Hydroxyl radical potentially destroys several biomolecules including proteins, DNA and membrane phospholipids. Due to the weakness of adjacent double bonds, polysaturated fatty acids of membrane phospholipids are sensitive to peroxidation to form lipid peroxy radical and subsequently lipid peroxide. Lipid peroxides are stable but their decomposition can be catalyzed by transition metals and metal

complexes to produce cyclic-peroxides and cyclic–endoperoxides, with fragmentation to aldehydes (e.g. malondialdehyde, MDA), conjugated dienes (4-hydroxyonenal, 4-HNE). This leads to the formation of covalent links to proteins or protein adducts and increased lysosomal fragility. The site of lipid peroxidation and whether the iron or hydroxyl radical is membrane-associated will affect the incidence of any free radical producing reaction (Bergamini, *et al* 2004). EDTA-Fe(II) complex does not diminish the reactivity of iron salts in the Fenton reaction and may catalyse the peroxidation. In comparison, DFO tightly binding to Fe^{3+} will completely inhibit lipid peroxidation in several systems.

1.2.12 Changes in plasma iron and NTBI formation in iron overload

Evidence for existence of NTBI

Evidence that iron may exist in plasma unbound to transferrin and may be toxic was first provided by Hershko (Hershkko, *et al* 1978). Despite considerable skepticism initially, the evidence for the existence of NTBI has now become persuasive. NTBI was firstly recognized as nonspecific iron present in thalassemic serum, which was chelatable and subsequently dialysable or filtrable. Previously described, bleomycin-reactive iron present in the plasma of iron overload patients can generate harmful hydroxyl radicals and promote lipid peroxidation. It has been proposed that NTBI is probably a low MW iron complex loosely bound to plasma proteins such as albumin. An independent study argued that such NTBI might be the feritin-bound iron. It has also been postulated that NTBI in plasma of hemochromatosis patients is mainly iron citrate and also iron-citrate-acetate complexes whereas NTBI in thalassemic serum comprises low MW iron complexes and iron loosely bound to serum proteins. Nevertheless, the precise nature of nonspecific NTBI has not been characterized so far (Breuer, *et al* 2000).

Mechanisms of NTBI toxicity

Previous evidence demonstrated that NTBI ultrafiltrate from hemochromatosis sera was capable of promoting the free-radical formation by xanthine oxidase and acetaldehyde, and stimulating the peroxidation of phospholipid liposomes. In the absence of detoxifying intracellular enzymes such as superoxide dismutase, catalase and gluthathione reductase, NTBI may promote the formation of hydroxyl radicals and accelerate the peroxidation of membrane lipids. NTBI also increases the formation of MDA and conjugated dienes and is associated with increased respiratory excretion of low MW alkanes (Sadrzadeh, *et al* 1997).

1.2.13 Iron status in end-stage renal disease (ESRD) patients

Iron deficiency

There are three important mechanisms that have been proposed to explain the high frequency of iron deficiency in hemodialysis patients.

1) Iron absorption in dialysis patients

Absorption of iron from the gastrointestinal tract is modulated by level of body iron stores, EPO and erythropoiesis. It takes places almost exclusively in the duodenum and proximal jejunum, and is strictly regulated depending on dietary intake, intraluminal factors, erythropoiesis, capacity of the intestinal mucosal cell and the tissue-iron storage level. Ingested iron is reduced to the ferrous form and bound to high MW chelators. Red meat contains primaliry heme iron which is more avidly absorbed. Restrictions in the ingestion of red meat, common in dialysis patients, may account so that the small amount of iron that is absorbed in this population. It should be noted that the proportion of ingested iron absorbed in individuals with normal renal function is also low, with 1 mg of iron or less absorbed daily. On the other hand, iron absorption generally increase in the face of accelerated erythropoiesis or a decrease in body iron stores. Conflicting data have appeared in the literature regarding the balance of these factors influencing iron absorption in dialysis patients. Early studies suggested that iron absorption was normal in patients on dialysis, whereas more ferrokinetic studies found that uptake of iron by intestinal mucosal cells and iron retention was decreased significantly in dialysis patients. High ferritin levels, which it can occur in dialysis patients despite low iron stores, may also impair the normal feedback that would increase absorption during deficiency states (Hughes, *et al* 1992).

2) External blood loss

Several factors contribute to ongoing blood loss in dialysis patients including blood retained in the dialyzer and blood tubing at the end of each dialysis treatment, frequent blood testing, occult blood loss and blood lost from puncturing or removing needles from hemodialysis vascular accesses. Approximately 1 to 3 g of iron is lost annually from these causes. Reasonably, the higher the hematocrit achieves with recombinant human erythropoietin (rHuEPO) administration, the greater the iron loss with each milliter of lost blood, because of the higher hematrocrit. The Anemia Work Group of the Dialysis Outcomes Quality Initiative suggested that 25 to 100 mg of iron would need to be replaced weekly in hemodialysis patients just to offset the iron lost because of the ongoing external blood losses. There are no data available on the external blood losses that occur in peritoneal dialysis patient. Clearly, the lack of blood loss in the dialyzer

and tubing, the absence of needle sticks, and heparin use would lead one to predict a substantially smaller blood loss in this patient population (Eschbach and Adamson 1999).

3) Functional iron deficiency

A further complicating feature in the management or identification of iron deficiency in the dialysis population is the introduction of the concept of functional iron deficiency. Functional iron deficiency is present when the usual tests for iron deficiency in dialysis patients do not indicate absolute iron deficiency (ferritin of more than 100 ng/ml, transferrin saturation of more than 20%). Patients respond to additional iron administration with a rise in hematocrit at a stable EPO or maintaining a stable hematocrit with a lower EPO. Patients with functional iron deficiency therefore have apparently insufficiently available iron to keep up with the demands of stimulated erythropoiesis that occur when exogenous EPO is administered (Tarng, *et al* 1999).

In some patients, the inability to mobilize iron rapidly relates to the presence of reticuloendothelial blockade. Dialysis patients may have other conditions that may preclude an appropriate response to iron therapy or prevent the use of iron stores that would be sufficient to sustain additional erythropoiesis. This effect may be caused by increased levels of circulating cytokines that are capable of inducing macrophages to more avidly take up and hold on to iron. The effect of cytokines may involve a decrease in endogenous EPO production or a decrease in responsiveness of EPO precursor cells to endogenous or exogenous EPO. Interleukin-1β ($IL_1\beta$) (possibly acting through interferon-γ, IF_γ) and tumor necrosis factor-α (TNF_α) can decrease the responsiveness to EPO as well. Such patients typically have the high levels of transferrin saturation and serum ferritin. Iron therapy in these patients with functional iron deficiency generally will not produce a favorable response (Eschbach and Adamson 1999).

Iron overload

Iron overload was a common complication in the patients with chronic renal failure treated with dialysis prior to the availability of rHuEPO therapy. Iron overload was the result of hypoproliferative erythroid marrow function coupled with the need for frequent blood transfusion to manage symptomatic anemia. The repetitive use of intravenous iron with or without the use of blood transfusions also contributed to iron deposition in the hepatic parenchymal and reticuloendothelial cells (Crowley, *et al* 1987). There were no abnormal liver function tests or evidence of cirrhosis unless viral hepatitis resulted from the transfusions. With rHuEPO therapy, the excess iron stores were shifted back into circulating red blood cells while the anemia was partially corrected, and red blood cell were lost from circulation during hemodialysis. After

several years of rHuEPO therapy, most hemodialysis patients required iron supplements to replace the continuing blood losses (Boran, *et al* 1993).

There has been much concern raised about the potential toxicity of chronic iron overload in ESRD patients. These concerns relate to the following possible complications.

1) Hepatic parenchymal iron deposition

Some chronically anemic adults with normal renal function and multiple RBC transfusions resulted in iron overload and hepatomegaly. Another anemic subject (with normal renal function) who received excessive amounts of intramuscular iron became iron overload. Iron overload in hemodialysis patients has resulted in increased iron deposition in hepatic parenchymal as well as Kufffer cells and also been associated with a proximal myopathy (Nomura, *et al* 1990). Ten patients had proximal muscle weakness and serum ferritin levels of 1030 to 5000 ng/ml. However, the amount of iron deposition did not correlate with the severity of muscle weakness. All of these patients had one or more of the "hemochromatosis alleles (HLA3, B7 or B14)" and were at increased risk to develop iron overload and muscle iron deposition. Among the thousands of dialysis patients, many would either have the disease or be a carrier of genetic disorder. Theoretically, these patients would lack the regulatory mechanism that prevents iron absorption in the presence of adequate iron stores, causing the preferential iron deposition in parenchymal cells, rather than reticuloendothelial cells. A previous study showed that dialysis patients who had hemochromatosis alleles were more prone to develop iron overload from blood transfusion than those patients without any of these HLA antigens. However, one contradictory result showed that there was not any correlation between the presence of hemochromatosis allele in ESRD patients and iron overload (Muller-Wiefel, *et al* 1981, Quereda, *et al* 1987).

2) Permanent organ damage

In dialysis patients with hemosiderosis in cirrhosis, pancreatic fibrosis, or cardiac failure caused by iron overload have not been reported. All of these complications are common in the dialysis population and are not necessarily related to iron overload. However, many dialysis patients who have the primary hemochromatosis gene are at risk to exhibit findings of this disorder eventually. One research report issued in Finland suggested that the excess body iron evaluated with high serum ferritin levels was a risk factor for myocardial infarction. Contradictorily, a large study from the United States concluded that higher transferrin-saturation levels were not associated with an increased risk of coronary artery disease (Eschbach and Adamson 1999).

3) Increased risk of bacterial infections

There is no free iron in circulation as long as transferrin is less than fully saturated. Most dialysis patients of 20 to 30 years ago who had iron overload had transferrin saturation of less than 95%. Parenteral iron-treated patients rarely had transferrin saturation chronically greater than 50%. Moreover, an increased incidence of infections has been reported in dialysis patients with iron overload, probably due to the suppression of phagocytosis by iron. Seifert and coworkers reported that hemodialysis patients (serum ferritin =1001-2000 ng/ml), who they had received multiple blood transfusions without desferrioxamine (DFO) chelation, had a significantly increased incidence of bacterial infections when compared with control hemodialysis patients (serum ferritin =10-330 ng/ml) (Seifert, *et al* 1987). Other hemodialysis patients treated with DFO (serum ferritin =1856-6112 ng/ml) also had a significantly increased incidence of bacterial infections compared with the control group. Probably, immunological suppressive effects of multiple blood transfusions could account for the increased susceptibility to infections. Anemia is associated with a greater incidence of infection, and rHuEPO reverses the leukocyte dysfunction in dialysis patients with iron overload (Peter 1983).

4) Increased free radical generation

There may be increased free radical generation from free iron that will cause oxidant injury. Transferrin that is normally less than 50 % saturated with iron transports iron from the gut to the marrow and RE cells and other iron requiring-tissues. However, if free iron is available, it can be reversibly oxidized or reduced. This iron form is potentially hazardous and able to participate in the generation of powerful reactive oxygen species (ROS), such as hydroxyl radical, thus causing cellular injury. However, most of iron in the body is bound to heme proteins (hemoglobin and myoglobin) or nonheme proteins (transferrin, ferritin and hemosiderin) and does not directly catalyze the generation of hydroxyl radicals or other oxidants. It is difficult to ascertain from the records of those dialysis patients with severe transfusion induced iron overload whether they exhibited evidence of tissue injury from iron overload (Eschbach and Adamson 1999).

In summary, iron overload without rHuEPO administration was a serious problem with hepatosplenomegaly, hypersplenism and hyperpigmentation commonly seen. There are not any data that iron overload from medicinal iron increases patients morbidity or mortality. The use of more intravenous iron to replace iron (blood) losses associated with the hemodialysis and to maintain optimal iron stores

should not pose a threat for the development of iron overload as long as the patient's iron status is monitored on a regular basis.

1.2.14 Non-transferrin bound iron in the plasma of hemodialysis patients

The anemia in ESRD patients can be treated by blood transfusion or successfully treated with rHuEPO. The efficacy of the latter treatment is determined by the availability of iron for erythropoiesis. In many hemodialysis patients, a (functional) iron deficiency limits the effect of the treatment. The response to rHuEPO is improved dramatically in numerous hemodialysis patients when iron is supplemented intravenously. Over saturation of transferrin during intravenous iron treatment represents the occurrence of circulating plasma iron that is not tightly bound to transferrin as "labile iron pool (LIP)" or "non-transferrin bound iron (NTBI). This form of iron would be potentially harmful and may act as a catalyst for the formation of extremely toxic hydroxyl radicals (Esposito, *et al* 2002).

1.2.15 Deferiprone

Deferiprone (1,2-dimethyl-3-hydroxypyridin-4-one, DFP, CP20 or L1) is the first oral iron chelator (MW 199) (Figure 1.8) used clinically, mainly in β-thalassemia patients. DFP was designed and firstly synthesized by Hider and Kontoghiorghes at King's College, University of London (Kontoghiorghes 1990). This chelator is a bidentate iron chelator that can bind to iron in a 3:1 ratio octahedral complex with a stability constant of 37, about six orders of magnitude higher than DFO. It is used in the removal of excess iron from the body, but may also be used in the detoxification of other metals such as aluminium in hemodialysis patients, plutonium in the nuclear industry and uranium in the military affairs.

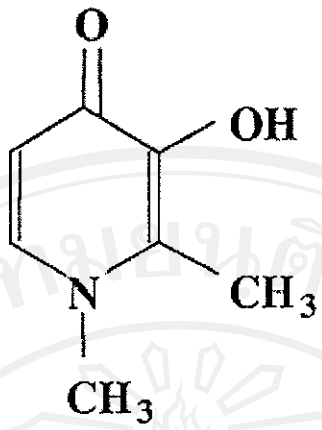


Figure 1.8 Structure of Deferiprone (From Chua et al., 2003).

Pharmacological activities of DFP

DFP is rapidly absorbed ($T_{1/2}$ estimated as 7-22 minutes), appears in plasma and is metabolized to a DFP-glucuronide conjugate that is incapable of binding iron. Free and iron-bound DFP are rapidly cleared from plasma with a $T_{1/2}$ of about 90 minutes; the glucuronide derivative is excreted more slowly with a $T_{1/2}$ estimated as 148 minutes. Following a single oral dose, only a small fraction of the total DFP is found in plasma after 12 hours, 1.3% for DFP, 5.3% for the DFP-glucuronide conjugate. Clearance of the DFP-glucuronide conjugate is slower in impaired renal-function patients. The drug is excreted in urine in free form, iron-bound form and DFP-glucuronide complexed with zinc or aluminium. About 80% of a single oral dose appears in urine within the first 24 hours. DFP probably induces less fecal iron excretion than DFO. Previous studies have shown a mean fecal excretion between 0 and 33% of a single oral dose of the drug, approximately between 0-6 mg in patients receiving 75–100 mg DFP/kg/day of the drug. The efficacy of the drug in the first 24 hours in iron loaded patients is about 4% assessed as [DFP-Fe]/[Total DFP] in the urine. DFP accumulates mostly in the liver, both in iron-loaded and normal rats. No excessive accumulation in any other organs including brain, bone marrow, endocrine organs, heart or thymus could be demonstrated, except for the gastrointestinal tract and kidneys, which are routes of excretion of DFP. Unlike DFO, DFP can remove iron from plasma transferrin. Approximately 20% of the iron excreted in the urine in heavily iron-loaded patients given a single dose of DFP may be derived from the transferrin bound-iron (Kontoghiorghes 1990, Stobie, *et al* 1993).

Clinical trials of DFP

DFP (100 mg/kg body weight) induced urine iron excretion (UIE) in iron-loaded patients approximately equivalent to that induced by a 12-hour subcutaneous infusion of 50 mg/kg DFO. Iron excretion is greater in the more iron-loaded patients and with larger doses of DFP. Total daily dose of drug given as two or three sub-doses throughout the 24 hours produce more UIE compared with a single large dose. The most widely used regime in long-term clinical trials is 25 mg/kg given three times daily. Patients usually take DFP every day of the week. Compliance with DFO is substantially poorer than with DFP. Serum ferritin levels in β-thalassemia major patients treated with DFP remain unchanged during 6–12 months of DFP therapy but significantly fall after 12 months or longer, particularly among those patients with the highest initial iron burden. A significant fall in NTBI has been shown after six months of DFP therapy in β-thalassemia patients. Most importantly, liver iron content (LIC) falls in those DFP-treated patients with the highest initial concentrations (Chua, *et al* 2003). The evidence that cardiac or endocrine iron is reduced by DFP chelation therapy in β-thalassemia major is now increasing. If there is a reduction of LIC, serum ferritin, UIE and NTBI, a reduction of iron content in the organs likely takes place. Regarding to thalassemia intermedia, one year of DFP therapy causes a reduction of cardiac iron measured by magnetic resonance imaging (MRI). Improvement in cardiac MRI findings among β-thalassemia major patients receiving DFP has also been mentioned. Anterior pituitary iron has also been fallen in two β-thalassemia major patients treated with DFP (Adhikari, *et al* 1995, Bartlett, *et al* 1990, Olivieri, *et al* 1993).

Adverse effects of DFP chelation therapy

The major adverse effects commonly are transient musculoskeletal and joint pains, neutropenia and agranulocytosis, gastric intolerance and zinc deficiency. Of these, agranulocytosis is the most disturbing and amounts to rendering the body's immune system fatally weak. The monitoring not only helps to safeguard the well being of the patient but also yields valuable data that can contribute to the development of a diagnostic method for agranulocytosis and improvement of the therapy with deferiprone (Adhikari, *et al* 1995, Bichile, *et al* 1993, Hoffbrand, *et al* 1998).

1.2.16 Curcumin

Historical background

Curcumin (diferuloylmethane) is a major yellow pigment isolated from the ground rhizome of the *Curcuma longa* Linn. in Family *Zingiberaceae*. As a powder, it is called turmeric and widely used as a spice and coloring agent in several foods such as curry, mustard, as well as in cosmetics and drugs (Figure 1.8).

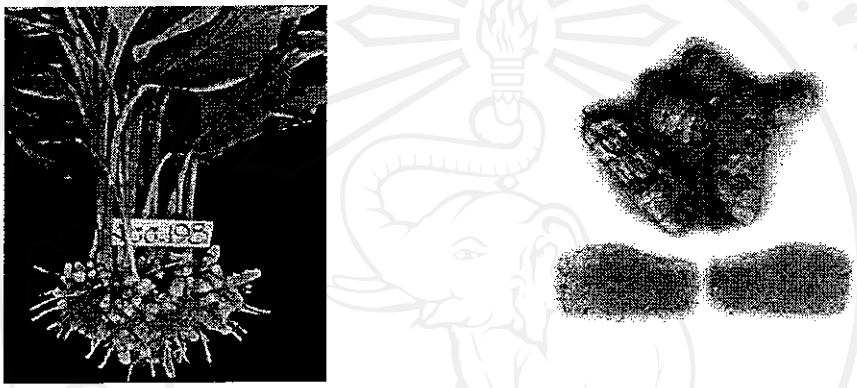
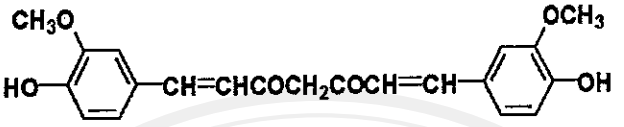


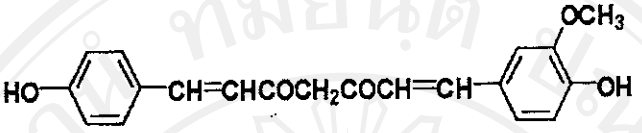
Figure 1.9 Turmeric plant and turmeric powder (From www.iisr.org).

Chemistry of curcumin

Three major curcuminoids namely curcumin, demethoxycurcumin and bisdemethoxycurcumin occur naturally in these *Curcuma* species (Figure 1.10 and Figure 1.11). The major constituent, curcumin, is the most important fraction of *C. longa* L. and its chemical structure was determined by Roughley and Whiting (1973). It melts at 176-177°C and forms red-brown salts with alkalis. Curcumin is soluble in ethanol, alkaline, ketone, acetic acid and chloroform; nevertheless, it is insoluble in water.



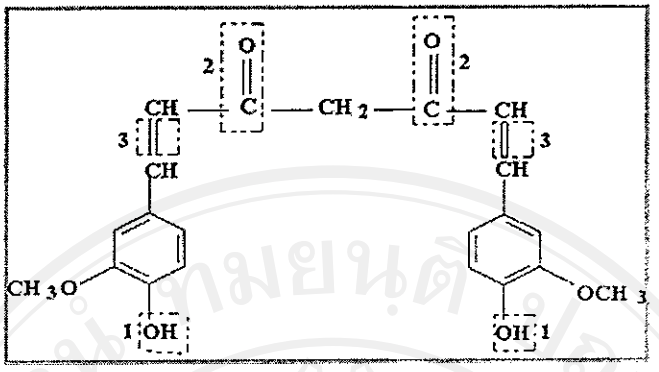
CURCUMIN Diferuloyl methane



DEMETHOXYCURCUMIN p-Hydroxy-cinnamoyl-feruloyl-methane

BISDEMETHOXYCURCUMIN pp'-Dihydroxy-dicinnamoyl-methane

Figure 1.10 Chemical structures of curcuminoids (From www.sabinsa.com).



1. Parahydroxyl groups

2. Keto groups

3. Double bonds

Figure 1.11 Chemical structures of curcumin (From www.sabinsa.com).

Metabolism of curcumin

Oral and intraperitoneal administration of [^{3}H] curcumin in rats led to fecal excretion of most of the radioactivity. In Figure 1.12, the major biliary metabolites were tetrahydrocurcumin-glucuronide and hexahydrocurcumin-glucuronide. A minor biliary metabolites was dihydroferulic acid together with traces of ferrulic acid. Considering the pharmacokinetics in mice, about 2.25 microgram/ml of curcumin (0.1 g/kg) administered intraperitoneally appeared in the plasma in the first 15 minutes then was taken up into liver cells for metabolic processing. Treatment of mouse plasma with β-glucuronidase can decrease the concentrations of two putative conjugates and concomitantly produce tetrahydrocurcumin and curcumin respectively. It is proposed that curcumin is biotransformed initially to dihydrocurcumin and sequentially to tetrahydrocurcumin with the catalysis of cytosolic reductase enzyme. Then these compounds were converted to monoglucuronide conjugates, dihydrocurcumin-glucuronide and tetrahydrocurcumin-gucuronide successively with the catalysis of microsomal uridine diphosphate- or UDP-glucuronyl transferase enzyme (Ravindranath and Chandrasekhara 1981).

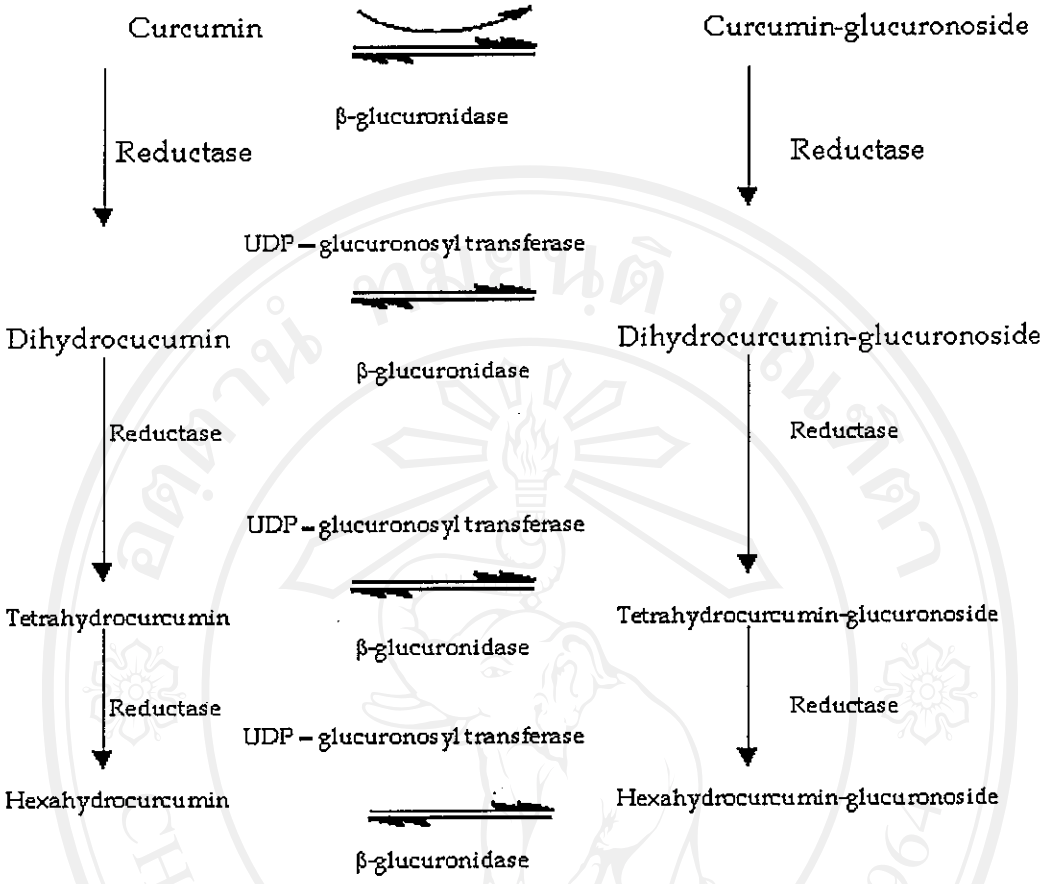


Figure 1.12 Proposed biotransformation and metabolism of curcumin in mouse plasma (From www. tetra hydrocurcuminoids.com).

Biological activities of curcumin

Curcumin has potent anti-oxidative, anti-inflammatory, anti-tumorigenic and anti-carcinogenic activities. These are the scavenge of free-radicals, inhibition of ornithine decarboxylase (ODC) gene expression and activity in urea cycle, inhibition of cyclooxygenase (COX) and lipoxygenase (LOX) activity in arachidonate cascade pathway, inhibition of carcinogen- DNA adduct formation and tumorigenesis in several animal models as summarized in Table 1.1

Table 1.1 Biochemical activities of curcumin

Biochemical activities	Reference
Scavenge superoxide anion and hydroxyl radical	(Kunchandy and Rao 1990)
Scavenge singlet oxygen	(Subramanian, *et al* 1994)
Inhibit lipid peroxidation	(Sreejayan and Rao 1994)
Inhibit TPA-induced ornithine decarboxylase (ODC) mRNA and activity	(Huang, *et al* 1988)
Inhibit TPA-induced skin inflammation	(Huang, *et al* 1997)
Inhibit lipoxygenase and cyclooxygenase activities	(Huang, *et al* 1991)
Inhibit arachidonic acid metabolism	(Conney, *et al* 1991)
Inhibit the formation of carcinogen-DNA adducts	(Conney, *et al* 1991)
Inhibit skin tumor initiation and promotion	(Huang, *et al* 1992)
Inhibit BaP induced forestomach and lung tumorigenesis	(Huang, *et al* 1994)
Inhibit ENNG-induced duodenal tumorigenesis	(Huang, *et al* 1994)
Inhibit azoxymethane-induced colon tumorigenesis in mice and rats	(Rao, *et al* 1995)

Structure-activity relationships

It is known that curcumin from *C. longa* L. belong to the class of curcuminoid and is very similar to diaryl heptanoids. Some authors demonstrated that the anti-inflammatory activity of curcumin and its derivatives comes from the hydroxyl and phenolic groups in the molecule, which they are essential for the inhibition of prostaglandins (PG synthetase) and leucotrienes (LT) (Iwakami, *et al* 1986, Kiuchi, *et al* 1982). Some suggested that the anti-inflammatory action is associated to the existence of the β-dicarbonylic

system, which has the conjugated double bonds (dienes), being responsible for this activity. This system seems to be responsible not only for anti-inflammatory power but also anti-parasitic activity (Araujo and Leon 2001). The presence of diene ketone system provides lipophilicity to the compounds, and thus probably better skin penetration. Curcumin is capable of scavenging oxygen free radicals such as superoxide anions and hydroxyl radicals. The phenolic and the methoxy group on the phenyl ring and the 1,3-diketone system seems to be important structural features that can contribute to these effects.

Iron-chelating activity of curcumin

Iron is present in excess or in forms where the electrons are incompletely coordinated. It has potential to participate in the generation of harmful free radicals, due to its facile ability to redox cycle between the Fe^{2+} and Fe^{3+} oxidation states. Curcumin and its derivatives are capable of free-radical scavenging, interacting with oxidative cascade, quenching oxygen, chelating and disarming oxidative properties of metal ions (Patro, *et al* 2002). Biological activity of curcumin has been attributed to hydroxyl group substituted on the benzene rings and also to the diketonic structure. The β-diketo moiety of curcumin undergoes a keto-enol tautomerism. Crystalographic studies have shown the symmetric structure of curcumin leads to a statistically even distribution of the enol proton between the two oxygen atoms. The strong chelating ability of diketones has been widely investigated towards a great number of metal ions; therefore curcumin could be of great importance in the chelating treatment of metal toxification and overload (Macro, *et al* 2002). Among all metals, iron earns a relevant position due to its indispensability in life, since it takes part in processes such as oxygen transport and electron transfer and DNA synthesis.

1.3 Objectives

1. To measure iron status and oxidative stress in ESRD patients who have received regular hemodialysis.
2. To quantify plasma NTBI in ESRD patients on regular hemodialysis.
3. To study the effects of hemodialysis on NTBI formation in ESRD patients on regular hemodialysis.
4. To examine the efficacy of deferiprone with curcumin for removing plasma NTBI *in vitro* in ESRD patients with regular hemodialysis and iron overload.
5. To investigate the preventive effects of curcumin on free radical formation in red blood cells induced by hydrogen peroxide.
6. To study the kinetic of iron binding with curcumin *in vitro*.