Chapter 4

Antioxidants as Complementary Medication in Thalassemia

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Additional information is available at the end of the chapter

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1. Introduction

Iron is an essential trace element found in all cellular forms of life and plays a crucial role in oxygen sensing and transport, electron transfer, and catalysis [1]. In ancient times, iron was used in medicine for the treatment of the following diseases; alopecia, acne, vesicular bollus, encrusting eruptions, erysipelas, paronychia, vaginal discharges, wounds, hemorrhoids, gout, tuberculosis, diarrhea, perianal fistulas, excessive lacrimation, vomiting, weakness, edema, fevers and cystitis [2]. Secondary iron overload in β-thalassemia patients is commonly caused by increased dietary iron absorption and can be a result of multiple blood transfusions [3]. Consequently, the excessive active iron catalyzes the production of a variety of reactive oxygen species (ROS), such as superoxide anions ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\bullet}$) via Haber-Weiss and Fenton reactions. The ROS is attributed to the function of the metal in the redox cycle and can damage many cells and tissues including the heart, liver, pancreas, erythrocytes and endocrine glands resulting in dysfunctions of the organs [4]. Hydrogen peroxide is normally applied to kill microorganisms in neutrophils; nevertheless, an excess amount can be toxic.

β-Thalassemia patients suffer from ineffective erythropoiesis and require regular blood transfusions to compensate for having chronic anemia. Transfused red blood cells (RBC) are taken up and degraded by the reticuloendothelial system (RES) or tissue macrophages, leading to a high accumulation of intracellular iron and saturation of plasma transferrin. The excess iron appears in successive forms of toxic iron as a labile iron pool (LIP), and non-transferrin bound iron (NTBI) and labile plasma iron (LPI) [5-7]. Effective iron chelators are required to remove the toxic irons in order to prevent oxidative damage in the vital organs, particularly the heart and liver. Iron chelators must be absorbed via the gastrointestinal tract into the blood circulation and target tissues readily, and show minimal side effects. They should complex

directly onto the plasma iron as well as the cellular iron; afterwards, the complexes can be excreted from the body easily [8]. Serum oxidant activity in young β-thalassemia major patients with iron overload is directly correlated with the serum ceruloplasmin and copper concentrations, and with serum iron (SI) concentration and total iron-binding capacity (TIBC), but not with serum vitamin E concentration [9]. Levels of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were greatly elevated in the RBC of β-thalassemia minor patients to fight cellular increased oxidant and are close to normal values in the RBC of β-thalassemia major patients due to the presence of transfused normal RBC [10]. Levels of antioxidant compounds such as serum retinol (vitamin A), carotenoids, α-tocopherol (vitamin E) were decreased in β-thalassemia major patients [11, 12]. Supplements of the antioxidant vitamins can prevent some of the damage in the thalassemic RBC membrane. Thus, antioxidant therapy can be a supplemental medical regime to meliorate pathophysiological complications and improve quality of life of thalassemia patients.

2. Iron overload in thalassemia patients

2.1. Causes of iron overload

Patients with β-thalassemia have a partial or complete lack of ability to synthesize the β-chains of hemoglobin [13]. This process of β-globin chain synthesis is controlled by gene that is found to be present in Chromosome 11. There are more than 200 points of mutation and rare deletions of this gene. The production of the β-globin chain can range from near normal to completely absent, leading to varying degrees of excess α-globins in the β-globin chain production. In the β-thalassemia trait, the one gene defect is asymptomatic and results in microcytosis and mild anemia. β-thalassemia major or Cooley anemia results from either severely reduced synthesis or the absence of synthesis in both genes. If the synthesis of the beta chain is less severely reduced, the person will have beta thalassemia intermedia. These persons experience symptoms that are less severe and do not require lifelong transfusions to survive past the age of 20 years [14]. The amount of iron (20 - 30 mg) required for the daily production of 300 billion RBCs is provided mostly by the iron that is recycled by the macrophages [6]. Importantly, iron stored in the macrophages is safe and does not lead to oxidative damage [15]. Iron overload can be caused by an increase in dietary iron absorption in hereditary hemochromatosis patients [16] and by multiple blood transfusions in β-thalassemia patients. Duodenal iron absorption in normal persons is approximately 1 – 2 mg/day and balanced with iron excretion at 1 – 2 mg/day. Though thalassemia intermedia patients do not receive transfusions, abnormal iron absorption produces an increase in the body's iron burden evaluated to 2 – 5 g/year (3 - 9 mg iron/day) [17]. Regular blood transfusions (420 ml/unit of donor blood equivalent to 200 mg of iron) lead to double iron accumulation. Increased iron absorption along with multiple blood transfusions in thalassemia patients can result in hemosiderosis, oxidative stress, hypercoagulability, liver inflammation, cardiomyopathy, endocrine dysfunctions and bone deformity, of which the complications can be meliorated by many drugs and agents (Figure 1).

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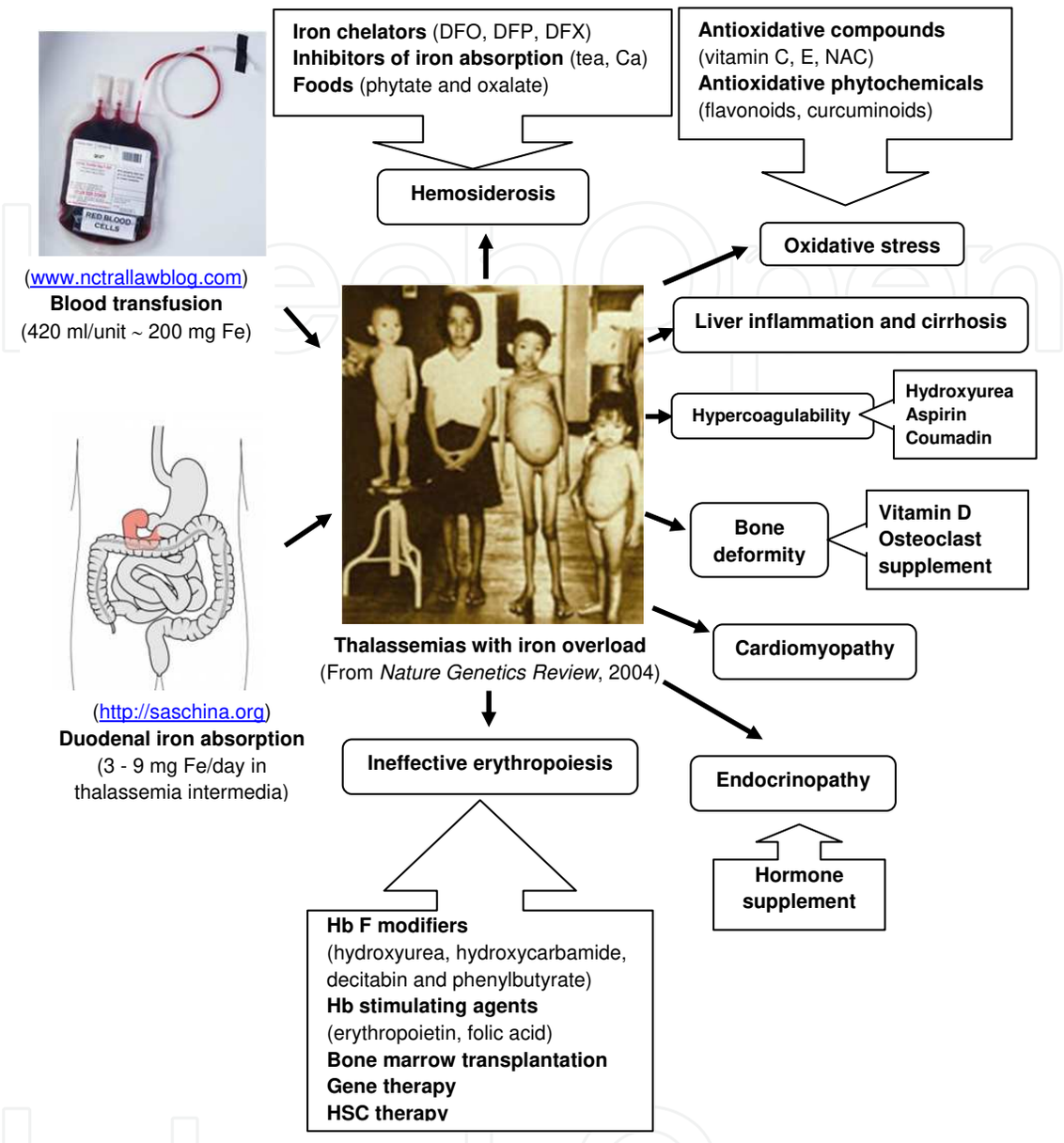

Figure 1. Complications and treatments of thalassemia patients with iron overload

Consequently, iron overload and accumulation introduces progressive damage in the liver, heart and in the endocrine glands. Plasma NTBI occurs in patients who have had multiple blood transfusions. NTBI was originally identified in reference [18] for playing a major role in the pathological conditions of iron overload. Circulating NTBI, as well as LPI, is detected whenever the capacity of transferrin to incorporate iron is derived either from the GI tract or when RES becomes a limiting factor. Both forms of toxic iron appear primarily in transfused patients where the TIBC has been surpassed [19]. Pathologically, the NTBI fraction seems to be translocated across the cell membrane irregularly, while the LPI is redox active and susceptible to chelation [20]. Chronic iron overload has been attributed to highly elevated levels of plasma iron and high accumulations of tissue iron. Excessive iron accumulation in

the vital organs is the cause of various liver diseases (e.g. hepatitis, hepatic fibrosis and hepatocellular carcinoma), cardiomyopathies (e.g. cardiac arrhythmia and heart failure) and endocrine gland dysfunction (e.g. diabetes, growth retardation, hypogonardism and hypoparathyroidism) [21, 22].

2.2. Chemistry of catalytic iron

Iron acts as a cofactor within the active site of key enzymes in the biochemical pathways and as a chemical catalyst in the unique redox activity. The iron can cycle between two oxidation states, ferric ion [Fe(III) or Fe^{3+}] and ferrous ion [Fe(II) or Fe^{2+}], allowing it to act as an electron donor and acceptor [23]. The biochemical functions of iron in vital cells are dependent on its chemical properties. Limitation of iron bioavailability under aerobic conditions occurs when the Fe^{2+} is rapidly oxidized in the solution to insoluble Fe^{3+} at physiological pH [$K_{\text{free Fe(III)}} = 10^{-18}$ M] [24]. Fe(III) iron is the most stable state of the biological complexes at physiological oxygen concentrations. Many complexes of iron and biomolecules (protein or simpler molecules) are involved with reduction potential or redox potential (E_h). The E_h of complexed iron in the range of a biological oxidant is +820 mV to the reductant at −320 mV, the redox reactions are reversible whereas the reaction may be irreversible for the iron complexes when the reduction potential occurs outside this range [1].

Iron is a crucial enzyme cofactor that acts in the reduction-oxidation reaction in the metabolism in the cells. Of outstanding interest, iron deposition in the heart cells can lead to oxidative stress and cellular damage [25-27]. Heart failure is the leading cause of death among hemosiderotic β-thalassemia patients, of whom, around 60% die of this cardiac failure [28-30]. The importance of metals to both enzymatic reactions and oxidative stress makes them the key players in mitochondria. Mitochondria are the primary energy-generating organelles of the cells that produce ATP through a chain of enzymatic complexes that require cytochromes iron and sulfur-iron, and are highly sensitive to oxidative damage. Moreover, the heart is one of the most mitochondria-rich tissues in the body, making metals of particular importance to cardiac function [31]. In cardiac cells, excess iron may result in oxidative stress and an alteration of myocardial function because of the DNA damage that is caused by hydrogen peroxide through the Fenton reaction [6, 32]. Harmful effects of iron overload on the hearts of patients with β-thalassemia major can be monitored [25, 33-38] and treatment with effective iron chelators can protect these patients from cardiac arrhythmia [39, 40]. Iron catalyzes the production of ROS including $O_2^{\bullet-}$, H_2O_2 and $^{\bullet}OH$ via Haber-Weiss and Fenton reactions (Figure 2).

$$O_2^{\bullet-} + H_2O_2 \xrightarrow{Fe\ /\ Cu} HO^{\bullet} + HO^{-} + O_2 \qquad \textit{Haber-Weiss reaction}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^{\bullet} + HO^{-} \qquad \textit{Fenton reaction}$$

Figure 2. Hydroxyl radical formation in metals-catalyzed Haber-Weiss and Fenton reactions [41]

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Another Fenton reagent can be hemichromes, which are a family of denatured methemoglobins [42]. Hydroxyl radicals might be most harmful to lipid, protein and DNA, which are the essential cell components (Figure 3). The •OH-induced membrane damage can be related directly to a membrane-associated Fenton reagent [43]. Oxidative cell damage has been attributed to the emergence of excessive levels of LPI that promote the production of ROS to a level that exceeds the cellular defense capacity [44].

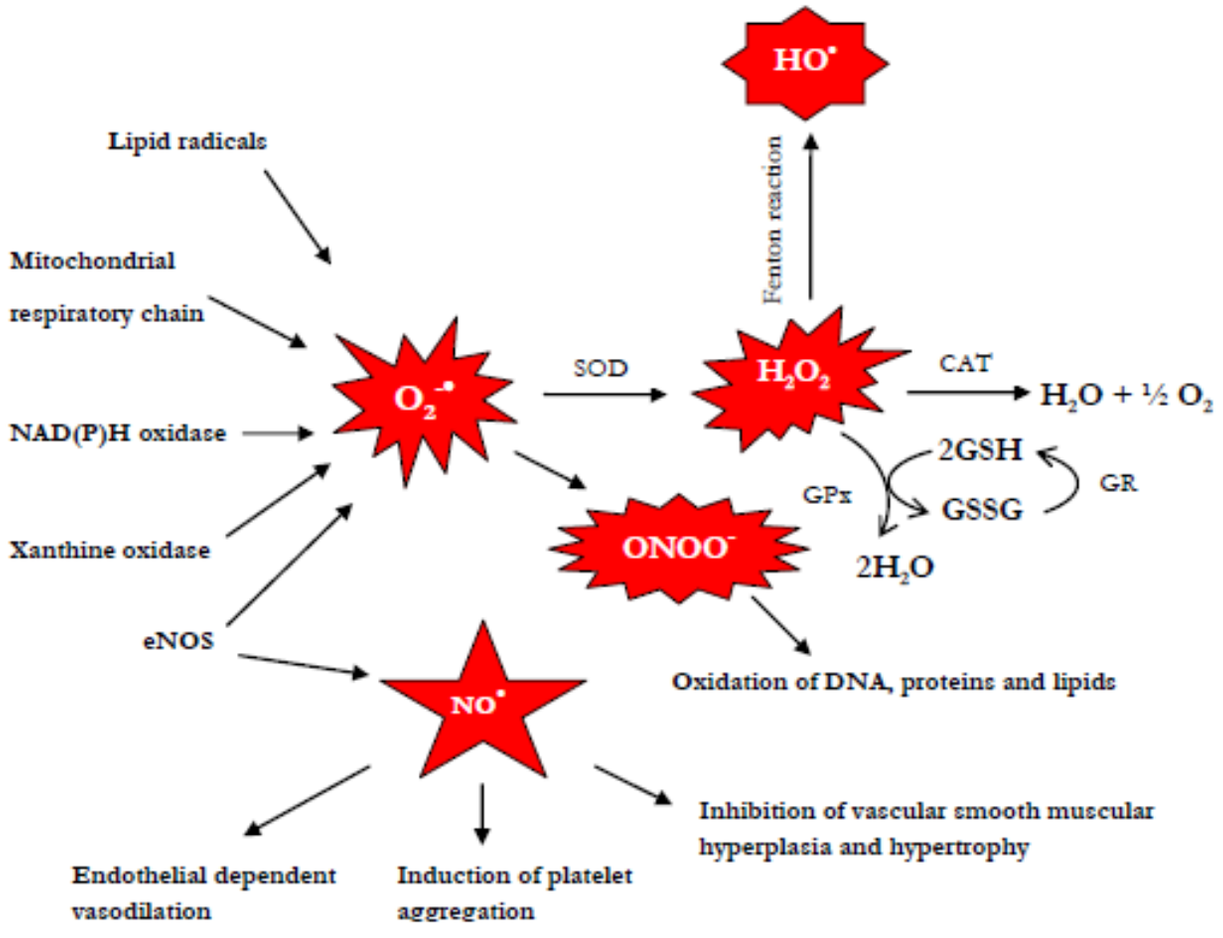

Figure 3. Production and harmfulness of ROS (Reprinted with modification from [45])

In β-thalassemia major patients, an outpouring of catabolic iron overwhelms the capacity of iron-transporting protein transferrin, and generates NTBI and redox-active LPI in the plasma compartment. Cytosolic LIP has been shown to be comprised of transitory Fe (II) and Fe (III) forms which are possibly mediated by specific cellular iron reductases [20, 44]. In sub-cellular organelles, mitochondrial iron serves in the formation of protein iron-sulfur clusters and porphyrins. Additionally, endosomal iron provides the translocation of the endocytosed iron

into cytosol or mitochondria, while lysosomal iron is associated with the products of iron-protein degradation [46, 47]. LIP is a source of chelatable and redox-active transient iron in the cells that serves as a crossroads of the cellular iron metabolism. The nature of the LIP has been revealed by its capacity to promote ROS generation in its "rise and fall" patterns. LIP plays a role as a self-regulatory pool that is sensed by cytosolic iron-regulatory proteins (IRP) and its feedback is regulated by an IRP-dependent expression of iron import and storage. The LIP can also be modulated by biochemical mechanism that override the IRP regulatory loops and contributes to basic cellular functions.

2.3. Oxidative stress in thalassemia patients

In one study, β-thalassemia children were found to have elevated levels of thiobarbituric acid reactive substances (TBARS), NAD(P)H oxidase (NOX) and SOD activity. Additionally, they were found to have decreased levels of CAT activity and reduced glutathione (GSH) concentration, along with unchanged GPx activity in their plasma compared to the plasma of the controls' [48]. Seminal SOD and CAT activities of homozygous β-thalassemic patients with iron overload were increased, probably due to a compensatory reaction to the persistence of high levels of ROS. Increased seminal lipid peroxidation could have contributed to the impairment of sperm motility [49]. Erythrocyte free reactive (non-heme) iron was significantly higher in β-thalassemia patients with HbE (30% >controls), which was associated with a high level of serum TBARS (86% >controls) [50]. Elevated serum ferritin showed a positive correlation with elevated levels of such liver enzymes as aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP), but not γ-glutamyl transferase (GGT), confirming hepatic iron overload. Serum ferritin also showed a positive correlation with elevated levels of plasma TBARS and SOD. Plasma TBARS concentrations were increased in patients with α-thalassemia trait, and increased to the highest level in Hb H disease patient. Plasma levels of vitamin A, C, and E were significantly decreased in α- and β-thalassemia patients [51, 52]. In addition, their RBC revealed lower levels of vitamin E, GSH, CAT and SOD [51].

Isolated Hb chains can behave as pro-oxidants that can trigger oxidation of low-density lipoprotein (LDL). Importantly, the descending order of the different Hb chains to the relative oxidation of LDL protein, Apoprotein B (ApoB) and lipid parts was: α-globin chains > β-globin chains > HbA. This indicates that the extracellular globin chains may be the trigger of the lipoprotein alterations observed in β-thalassemia patients [53] and suggests that iron overload should be involved in the oxidative stress shown in the cells [54]. In comparison with apoprotein E2 (apoE2) and apoprotein E3 (apoE3) apoprotein E4 (apoE4) is considered the least efficient under conditions of oxidative stress in thalassemia patients and this imples that apoE4 is a genetic risk factor for left venticular dysfunction [55]. Mean triglyceride concentrations were not significantly different between thalassemia patients and the controls. Total cholesterol and LDL-cholesterol concentrations were found to be lower in β-thalassemia major and thalassemia intermediate patients than in the controls (p <0.001), while HDL-cholesterol concentrations were lower in thalassemia intermediate patients (p <0.03) [56]. This may account for increased erythropoiesis and cholesterol consumption in thalassemia intermediate patients, and iron overload and oxidative stress in β-thalassemia major patients. A previous

revision suggested that iron-induced free radical formation in thalassemia patients might lead to lipid peroxidation, LDL oxidation, stimulation of apoptosis and other damaging processes. The essence in the chelating and antioxidant treatments of thalassemia patients has to be considered within the context of free radical damage and its prevention [57].

Afanas'ev identified the major routes of superoxide damaging effects in the mitochondria. These include initiation of apoptosis through a reduction of cytochrome c, activation of uncoupled proteins by superoxide and a competition between superoxide and nitric oxide at the Complex IV site (or cytochrome oxidase). The author suggests an application of effective free radicals scavengers (rutin and flavonoids) for the treatment of thalassemic patients [58, 59]. From a comprehensive study in Indonesia, non-transfused thalassemia intermedia patients showed mild signs of oxidative stress and increased hemoglobin degradation, but revealed no significant indication of tissue or cell damage. Transfusion-dependent β-thalassemia major patients showed a highly significant decrease in antioxidants and thiols, and a tremendous iron overload along with cell damage. This situation was made even worse in long-term transfused patients [60]. ROS and lipid peroxidation were found to be higher, and GSH lower, in thalassemic RBC compared with normal RBC at the baseline as well as following the hydrogen peroxide treatment. These effects were reversed by treatment with antioxidative *N*-acetylcysteine (NAC) [61]. Platelets obtained from β-thalassemia patients contained higher ROS and lower GSH contents than those from normal donors, indicating a state of oxidative stress. Exposure of platelets to oxidants such as hydrogen peroxide and tert-butylhydroperoxide, or to the platelet activators (e.g. thrombin), ionophore (e.g. valinomycin) or phorbol myristyl acetate (PMA), stimulated the platelets' oxidative stress. Iron, hemin and thalassemic RBC also stimulated the platelets' oxidative stress. Therefore, oxidative stress of the platelets can lead to the activation of thromboembolic events [62]. Basal ROS level expressed as mean fluorescent intensity (FI) was higher in thalassemia polymorphonuclear cells (PMN) (FI = 95.6±19.8) than in normal PMN (FI = 12.7±4.5). Treatment of thalassemia PMN with PMA markedly increased the basal ROS level; in comparison, treatment with antioxidants, such as NAC, vitamins C and vitamin E, reduced their basal ROS but enhanced their PMA response. Administration of effective antioxidants may compromise their antibacterial capacity and give prophylaxis for recurrent infections [63].

DNA damage can be caused by iron-induced free radicals in thalassemia patients. Lymphocytes from a normal female did not respond to ferric chloride or hemosiderin, but did to ferrous chloride and ferrous sulphate. Comparatively, lymphocytes from an Australian patient with Hb S/β-thalassemia (double heterozygote-sickle phenotype) were more sensitive to ferrous sulphate treatment. Interestingly, desferrioxamine (DFO) and deferiprone (DFP) reduced the response [64]. Erythrocyte GPx activity was significantly lower in non-chelated β-thalassemia major patients than those chelated with either DFO or DFP [65]. β-Thalassemia major patients were deficient in vitamin A, C, D, B and folic acid [66]. Levels of ceruloplasmin concentrations and its ferroxidase activity were significantly higher in the thalassemia patients than in the healthy controls. Interestingly, the levels were significantly higher in thalassemia patients with Hp2-2 phenotype than in patients with other phenotypes, suggesting that thalassemia patients with Hp 2-2 phenotype are under greater iron-driven oxidative stress [67]. Reduction of delta-aminolevulinic acid synthase (δ-ALA) activity and an increase of peroxiredoxin-2 expression in thalassemic erythroid cells might represent two novel stress-response protective systems [68].

2.4. Iron chelation therapy

Nowadays, DFO, DFP and deferasirox (DFX) are iron chelators of choice used for the treatment of β-thalassemia patients with iron overload. Their chemical structures are shown in Figure 4 [69-72]. DFO (Desferal®) is the first drug that was introduced in the 1970s to treat iron overload. The hexadentate chelator has an extremely high affinity for iron (III) (DFO : Fe = 1:1, $K_a = 10^{29}$) and a much lower affinity for other metal ions, such as zinc, calcium and magnesium [73]. DFO is poorly absorbed from the GI tract and rapidly excreted in the urine (plasma half-life of 5 - 10 minutes), it must be therefore administered parenterally; intravenously (*iv*), intramuscularly (*im*) or subcutaneously (*sc*) [8, 74]. However, the drug exhibits side effects including an elevated body iron burden, serious neurotoxicity and abnormalities of cartilage formation [75-78]. DFP (L1 or Ferriprox®), a synthetic bidentate chelator, has been the first orally active drug available for clinical use. A previous study demonstrated DFP decreased serum ferritin and liver iron concentrations in transfusion-dependent thalassemia patients [79]. Using magnetic resonance imaging (MRI) technique, DFP was able to reduce cardiac iron overload and improve cardiac function more effectively when compared to patients treated with DFO [80]. Recently, the Government Pharmaceutical Organization (GPO) of Thailand has manufactured and launched domestically produced DFP product (GPO-L-One®) for the treatment of Thai thalassemia patients with iron overload. This will make the in-house DFP cheaper and more available than the imported DFP. However, its side effects include nausea, vomiting, gastrointestinal disturbance, leucopenia and thrombocytopenia and zinc deficiency, as they are typically observed these side effects are being evaluated in the patients [81, 82]. DFX (ICL670 or Exjade®), a tridentate oral chelator with a high affinity and specificity for iron, has been clinically used for the treatment of transfusion-dependent thalassemia patients since 2003 [83-85]. Efficacy and safety of DFX uses have previously been evaluated and reported [83, 84, 86-88]. Common side effects of DFX are abdominal symptoms (usually diarrhea), skin exanthems, elevated serum creatinine levels and renal tubular dysfunction [70].

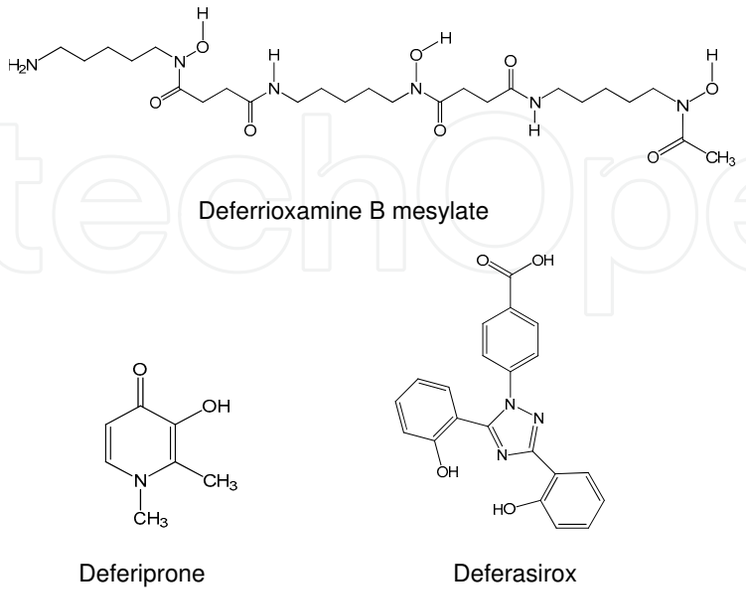

Figure 4. Chemical structures of DFO, DFP and DFX (Redrawn with modification from [89])

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In the medical regimen, DFO must be subcutaneously infused in β-thalassemia patients for extensive periods in order to achieve a negative iron balance, ranging from 8 to 12 hours, five to seven times per week, and at a daily dosage of 20 to 60 mg/kg body weight [72]. The patients experienced pain and swelling at the injection site, cumulatively leading to poor patient compliance [89, 90]. DFO chelation along with DFP or DFX has been designed to improve the efficacy and to diminish the adverse effects in the treated patients [91, 92]. Ideally, the iron chelator should be orally active, cheap and highly specific for iron, but not for other metal ions, and should freely penetrate into the target tissues, so as to get the patients compliant and show minimal side effects. Chelators can act upon different iron pools, including transferrin-bound iron (TBI), NTBI and LPI in plasma compartment, and LIP in cytoplasm to form iron-chelate(s) which will then be excreted in the urine and feces [8]. Clinical efficacy of chelation therapy has been evaluated at different periods of time, generally by following up the levels of the biochemical markers, including ferritin, total iron, TIBC, transferrin saturation, NTBI and LPI in plasma with colorimetry [93, 94], and of tissue biopsied iron with pathological examination (Perl's staining), as well as in organ iron with a semi-quantum uncoupled inductive device (SQUID) and magnetic resonance imaging (MRI) techniques [95-98]. Encompassing forms of plasma NTBI are readily chelated by effective iron chelators [19]. However, the pathologically relevant fraction of NTBI is that which is seemingly translocated across cell membranes in a non-regulated manner and leads to excessive iron accumulation in the liver, heart, pancreas and endocrine organs [20]. The LPI fraction is not only an accessible diagnostic marker of iron overload and cell toxicity, but also a clinical parameter for assessing the mode and efficacy of chelation therapy.

3. Antioxidants

Antioxidants can be defined as compounds that inhibit or delay, but do not completely prevent oxidation. There are two basic categories of antioxidants, namely synthetic and natural antioxidants.

3.1. Synthetic antioxidants

Mostly, the synthetic antioxidants that are widely used are phenolic compounds; for example, butylated hydroxyanisole, butylated hydroxytoluene, tertiary-butylhydroquinone and gallic acid (GA) derivatives.

3.2. Natural antioxidants

Natural antioxidants are found to be present in many sources such as plants, fungi, microorganism and even animal tissues. Phenolic compounds are also the majority group of natural antioxidants. The three important groups of antioxidant are tocopherols, flavonoids and phenolic acid. Natural antioxidants have been widely used in complementary and alternative medicines; in comparison, the synthetic antioxidants have reported signs of toxicological

evidence and caution should be imposed in their use. Most importantly, some natural antioxidants are more potent, efficiency and safer than synthetic antioxidants.

Synthetic antioxidant	Natural antioxidant
Inexpensive	Expensive
Widely applied	Use restricted to some products
Medium to high antioxidant activity	Wide-ranging antioxidant activity
Increasing safety concerns	Perceived as innocuous substances
Use banned in some cases	Increasing use and expanding application
Low water solubility	Broad range of solubility
Decreasing interest	Increasing interest

Table 1. Advantage and disadvantage of natural and synthetic antioxidants.

Advantages and disadvantages of the antioxidants are mentioned in Table 1. Most natural antioxidants are obtained in the diet from natural sources, especially from food of plant origin. Vegetables, fruits, and other foodstuffs are the best sources of these natural antioxidants. These antioxidants include vitamin C, vitamin E, carotenoids and polyphenol compounds. Antioxidants that are derived from natural sources are preferred by consumers. This is due to concerns over the toxic and carcinogenic effects of the synthetic antioxidants. Phenolic compounds include a large class of phytochemicals with interesting biological properties. Recently, the roles of these natural compounds in counteracting the negative effects of ROS/RNS and maintaining the redox homeostasis of biological fluids have been reported. Commonly, antioxidants can neutralize potentially harmful ROS in the cells before they induce lipid and proteins oxidation. Antioxidants from plants are believed to be useful in preventing aging, atherosclerosis, cancer, peptic ulcer, liver diseases and other degenerative pathologies, such as cancer, diabetes, Alzheimer's and Parkinson's diseases.

Antioxidants are any substances that significantly delay or inhibit oxidation of the oxidized substrate. These can greatly reduce the adverse damage to oxidants by crumbling or scavenging free radicals before they react with biological targets such as biomolecules, subcellular organelles, cells and tissues. The defense systems against the damage induced by ROS/reactive nitrogen species (RNS) fall into three categories: 1) preventive antioxidants that suppress free radical formation, 2) radical-scavenging antioxidants that inhibit initiation of chain reactions and intercept chain propagation and repair processes, and 3) adaptation to generate and transfer appropriate antioxidant enzymes. The hydrophilic antioxidants include vitamin C, uric acid, bilirubin, albumin, and the lipophilic antioxidants include vitamin E, ubiquinol and carotenoids. The radicals-scavenging antioxidant is described as a primary antioxidant, such as flavonoids and vitamin E (α-tocopherol), and the others that do not involve direct scavenge radical are secondary antioxidants.

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4. Potential antioxidants for use in thalassemias

Oxidative stress is not only a simple imbalance between the production and scavenging of ROS, but also a dysfunction of the enzymes involved in ROS production. Dysfunction of NOX, uncoupling endothelial nitric oxide synthase (eNOS), activation of xanthine oxidase (XO) and dysfunction of the mitochondria are underlying signs of oxidative stress; therefore, NOX is one of important therapeutic targets [99]. Pharmacological agents and approaches, as shown in Figure 5, can be implemented to relieve oxidative stress by the following mechanisms.

1) Inhibition of NAD(P)H oxidase activity:

1.1) Apocynin (methoxy-substituted catechol) is a natural compound that has a structure related to vanillin and exhibits anti-inflammatory activity, blocks an assembly of $p47^{phox}$ onto membrane complex, decreases production of superoxide, enhances production of nitric oxide and disrupts active NOX complex [100].

1.2) Chimeric peptides, such as polyethylene glycol-superoxide and NOX inhibitory peptide (gp91dstat), can inhibit association of $p47^{phox}$ with $gp91^{phox}$ [101] and synthesis of Angiotensin converting enzyme II (ACE-II) a dipeptidyl carboxy-peptidase that generates the vasoconstricting peptide angiotensin II [102].

1.3) Compound S17834 (e.g. benzo(b)pyran-4-one) inhibits NOX activity and superoxide radical production and attenuates atherosclerotic lesions in apolipoprotein-E-deficient mice [103].

1.4) Statins (e.g. simvastatin) are hydroxymethylglutaryl CoA (HMG CoA) reductase inhibitor hypocholesterolemic drugs that can prevent production of hydrogen peroxide and superoxide radicals, interfere with the renin-angiotensin-aldosterone system, and inhibit NOX activation and expression [99]. Currently, Zacharski and colleagues have demonstrated that statins increased HDL/LDL ratios and reduced serum ferritin levels in subjects with advanced peripheral arterial disease. The results imply that statins may improve cardiovascular disease (CVD) outcomes, possibly by countering the pro-inflammatory effects of excess iron stores [104].

1.5) ACE-II inhibitors (e.g. captopril, enalapril and quinapril) may act as antioxidants and down-regulate the vascular NOX system. Angiotensin II, through Angiotensin I (AT1) receptor activation, can induce vasoconstriction, cell growth, actions of pro-inflammatory cytokines and profibrogenic agents, and the production of vascular ROS, such as superoxide radicals [105]. Hence, the ACE-II inhibitors would lower ROS production and prevent vascular and renal changes.

1.6) AT1 receptor antagonists (e.g. candesartan, losartan and irbesartan) reduce the expression of NOX enzyme components, resulting in lower superoxide levels and increased nitric oxide bioavailability in rat blood vessels [106].

1.7) Other blockers including calcium channel blocker (e.g. amlodipine, nifedipine and dihydropyridine), β-blocker (e.g. metoprolol, propranolol and nebivolol) and α-receptor blocker (e.g. doxazosin) can inhibit or interfere with functions of NOX activity [106, 107].

2) Vitamins and dietary antioxidants (e.g. vitamin C, vitamin E, β-carotene, polyphenols, flavonoids, olive oil and nuts) can decrease the oxidation of LDL (bad cholesterol), improve vascular endothelial functions, enhance NOS activity and attenuate NOX activity in rat aorta.

3) L-Arginine is a nitric oxide generator that can improve vascular function and regulate NOS expression and synthesis.

4) Thiol-containing compounds (e.g. α-lipoic acid and NAC) potentially inhibit LDL oxidation, decrease oxidative stress, and attenuate hypertension, insulin resistance and oxidative stress.

5) Estrogen and hormone replacement therapy can reduce the morbidity and mortality associated with CVD, lower production of superoxide radicals, up-regulate NOS gene expression, activate cyclo-oxygenase (COX) gene and reduce the production of vasoconstrictor endothelin.

6) Cu/Zn SOD (MW = 31 kD) mimetics are selective synthetic compounds; for example, M40401 (MW = 483), M40403 (MW = 501), SC-55858, that either inhibit their formation or remove superoxide anion [108].

7) Xanthine oxidase inhibitors (e.g. oxypurinol and allopurinol) improve endothelial function in hypercholesterolemic subjects, type II diabetic mellitus (DM) patients, congestive heart failure (CHF) and cigarette smokers.

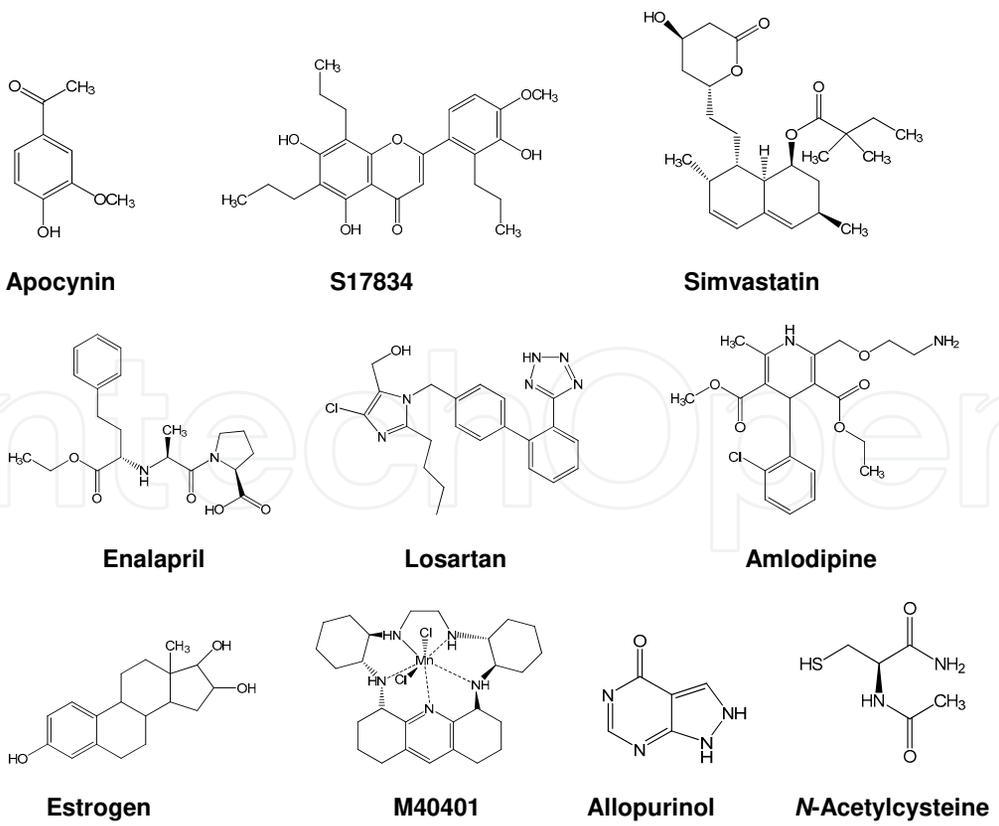

Figure 5. Drugs used for therapeutic purposes exhibits antioxidant activity

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5. Benefits of antioxidants in thalassemia patients

5.1. Administration in thalassemia patients

β-Thalassemia major patients who suffered from leg ulcers and were treated with ascorbic acid (3 g/day) for eight weeks showed a high rate of either complete or partial healing [109]. Improvement of ulcer healing in β-thalassemia major patients was observed when they were orally administered with pentoxifylline (1.2 g/day) which is xanthine derivative and functions as a competitive non-selective phosphodiesterase inhibitor [110]. Plasma antioxidant enzyme activity and selenium concentration increased in subjects with the Hb Lepore trait and were found to be significantly low in those patients with the α-thalassemia trait, while erythrocyte Se content was significantly increased in α-thalassemia patients [111]. *N*-allylsecoboldine may act as an effective antioxidant and protect cells against oxidative damage in β-thalassemic red blood cells [112]. Plasma vitamin E levels were lower in β-thalassemia intermedia patients compared to the controls and these levels were positively correlated with vitamin E in the LDL [113]. Treatment of the patients with vitamin E (600 mg/day, orally) for nine months improved the antioxidant/oxidant balance in the plasma, LDL particles and red blood cells, and counteracted the lipid peroxidation processes [114].

Significant decrease of GSH and proliferation in peripheral blood mononuclear cells (PBMC) were found in β-thalassemia major patients, probably due to the abnormality of cell mediated immunity (CMI) under iron overload conditions. Treatment of the patients with silymarin, which is an extract of milk thistle seed containing anti-hepatotoxic silibinin, led to a restoration of the GSH levels and PBMC proliferation, suggesting antioxidant and immunostimulatory activities and the benefits of using flavonoids to normalize immune dysfunction in β-thalassemia major patients [115]. *In vitro* treatment of blood cells from β-thalassemia patients with *N*-acetylcysteine amide (AD4), NAC and vitamin C increased the reduced GSH content of RBC, platelets and PMN leukocytes, and reduced their ROS. Intra-peritoneal injection of AD4 to β-thalassemic mice (150 mg/kg) reduced the parameters of oxidative stress ($p < 0.001$). These may imply the superiority of AD4, compared to NAC, in reducing oxidative stress markers in thalassemic cells, both *in vitro* and *in vivo*, and also providing a significant reduced sensitivity of thalassemic RBC to hemolysis and phagocytosis by macrophages [116]. *In vitro* treatment of blood cells from β-thalassemia patients with fermented papaya preparation (FPP) increased the GSH content in the RBC, platelets and PMN leukocytes, and reduced their ROS, membrane lipid peroxidation and phosphatidylserine (PS) externalization. Importantly, oral administration of FPP to β-thalassemia mice (50 mg/mouse/day for 3 months) and to thalassemia patients (3 g x 3 times/day for 3 months) reduced the levels of the oxidative stress parameters significantly. Suggestively, the FFP would be beneficial in reducing thalassemic RBC sensitivity to hemolysis and phagocytosis by macrophages, improving PMN ability to generate the oxidative burst and to reduce the platelet tendency to undergo activation [117].

Treatment of thalassemic RBC with erythropoietin (Epo) increased their GSH content and reduced their ROS, membrane lipid hydroperoxides, and PS exposure. Injection of Epo into heterozygous β-thalassemia mice reduced the oxidative markers. Probably, Epo might likely be an antioxidant that can alleviate symptoms of hemolytic anemia and reduced susceptibility

of RBC to undergo hemolysis and phagocytosis [118]. Thalassemic lymphocytes exhibited approximately a two-fold increase in the sensitivity to treatment of food mutagen/carcinogen, 3-amino-1-methyl-5H-pyrido(4,3-b)indole (or Trp-P-2) *in vitro*. However, treatment with flavonoids (quercetin and kaempferol) reduced the responses to Trp-P-2 to untreated control levels, significantly [119]. Transfusion-dependent thalassaemia patients were vitamin E deficient (0.45±0.21 mg/dl in all patients, 0.43±0.19 mg/dl in splenectomized patients), when compared to healthy subjects (0.76±0.22 mg/dl). Replacement therapy with vitamin E is necessary to correct its low serum levels easily (0.36±0.13 mg/dl before therapy and 1.19±0.35 mg/dl after therapy) [120]. Paraoxonase (PON) and arylesterase activities were significantly lower in β-thalassemia major patients than in the control healthy subjects, whereas total oxidant status, total peroxide concentration levels, and the oxidative stress index were significantly higher in the patients than in the controls in reference [121]. In addition, the activity of prolidase, a hydrolytic enzyme involved in collagen degradation was significantly increased in β-thalassemia major patients (53.7±8.7 U/l) compared to the control group (49.2±7.2 U/l). Total oxidant status was significantly increased in the patients (5.31±3.14 mmol H_2O_2 equivalent/l) compared to the controls (3.49±2.98 μmol H_2O_2 equivalent/l). Oxidative stress index was significantly increased in the patients (3.86±3.28 arbitrary unit) compared to the controls (2.53±2.70 arbitrary unit), while antioxidant capacity expressed as TEAC (1.61±0.30 μM) in the patients' plasma and this was not significantly different from that (1.64±0.33 μM) of the controls' plasma [122]. Administration of lipophilic antioxidant vitamin E (10 mg/kg/day for 4 weeks) is beneficial in the management of transfusion-dependant β-thalassemia HbE patients [123]. A current study has demonstrated that levels of iron parameters, such as serum ferritin, NTBI and transferrin receptors, were significantly increased in β-thalassemia major and thalassemia intermedia patients, compared to the controls and in severe cases when compared to the mild cases. Levels of serum ferritin, MDA, NTBI and GSSG/GSH were significantly increased in thalassemia intermedia patients; activities of serum glutathione reductase (GR), GPx and SOD were significantly reduced in these patients; while serum ascorbic acid concentrations were mildly reduced in the patients [124]. Interestingly, DFP has been reported to be a potent pharmaceutical antioxidant [125].

Patients with Hb H disease, β-thalassemia/Hb E and β-thalassemia major had vitamin E deficiency; however, supplements of vitamin E and selenium to the patients prevented RBC oxidation and increased RBC resistance to oxidative damage [126, 127]. There were no significant differences in the mean serum vitamin A, E concentrations and the vitamin E/cholesterol ratio between pregnant women with normal hemoglobin and hemoglobinopathies (Hb E and thalassemia) [128]. Patients with β^0-thalassemia/Hb E disease with lower blood Hb concentration had significantly higher erythrocyte SOD activity and the Hb concentrations were inversely correlated with the SOD activities (p <0.001) [129]. Supplementation of vitamin C plus vitamin E has greater benefits than vitamin E alone in promoting antioxidant status [130]. Administration of CoQ_{10} (100 mg/day) for six months markedly increased activities of plasma SOD, CAT and GPx, and decreased plasma MDA concentration of β-thalassemia HbE patients [131]. Increased oxidative stress in β-thalassemia/Hb E patients relates to higher levels of MDA, SOD and GPx in RBC, serum NTBI, and lower level of RBC GSH.

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5.2. Intervention of antioxidants in β-knockout (BKO) thalassemia mice

Nowadays, all clinical trials in humans and animals need to be approved by highly experienced ethical committees. Accurate animal models that recapitulate the phenotype and genotype of patients with β-thalassemia would enable researchers to develop possible therapeutic approaches. In this case thalassemic mice have been developed by groups of researchers [132-135]. The Thalassemia Research Center at Mahidol University, Salaya Campus in collaboration with the Murdoch Children Research Institute Melbourne, Australia have inbred wild type (WT) C57BL/6, β-knock (BKO) thalassemia ($Hb\beta^{th\text{-}3}/Hb\beta^{+}$), β-thalasssemia/HbE ($LCR\varepsilon^{G}\gamma^{A}\gamma\delta\beta^{E}$) $Hb\beta^{+}/Hb\beta^{+}$) (HT HbE), double heterozygous ($LCR\varepsilon^{G}\gamma^{A}\gamma\delta\beta^{E}$) $Hb\beta^{+}/Hb\beta^{th\text{-}3}$) (DH) and rescued β-thalassemia Hb E ($LCR\varepsilon^{G}\gamma^{A}\gamma\delta\beta^{E}$) $Hb\beta^{0}/Hb\beta^{0}$) (rescued β/HbE) mice in order to investigate the properties of the potential antioxidants and iron-chelating agents.

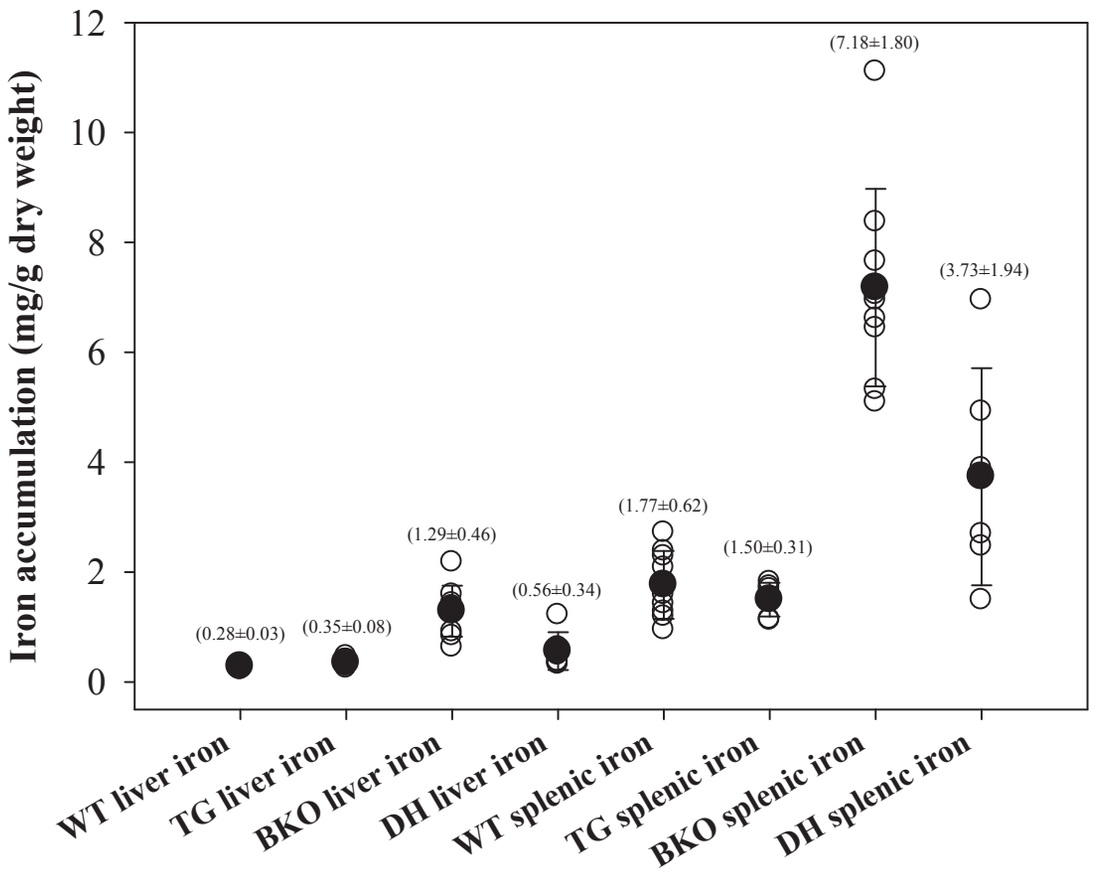

Figure 6. Iron deposition in the livers and spleens of the wild type (WT), transgenic (TG), β-knockout (BKO) thalassemia and double heterozygous (DH) thalassemia mice (S. Srichairatanakool, unpublished data)

Having measured the amounts of tissue iron of the WT and thalassemia mice, the BKO mice had the highest iron content accumulated in the livers and spleens when compared to the TG mice and WT mice, which the splenic iron content was far higher than the liver iron content (Figure 7). The results indicated that anemic conditions persisting in the BKO mice were similar to β-thalassemia intermedia and enhanced an increase of duodenal iron absorption for accelerating erythropoiesis. In our experimentations, feeding the BKO mice with a high iron diet (such as iron ferrocene) can load iron into their blood and tissue compartments, leading to iron overload and oxidative tissue damage. Their hematological parameters were determined and are illustrated in Table 2 [136]. Most importantly, the BKO thalassemia mice were found to be the most anemic when compared to the other types of thalassemia mice and their phenotype mimiced the human thalassemia intermedia patients. The mice heterozygous for deletion of the β-globin gene appear normal, but their hematologic indices show the characteristics that were typical of thalassemia intermedia. These include dramatically decreased hematocrit (Hct), hemoglobin (Hb) and red blood cell counts [136]. Bone deformities and splenic enlargement due to increased hematopoiesis [137], and iron overload in the vital organs (e.g. spleen, liver and kidneys) were also found in the heterozygous β-thalassemic mice, in reference [138].

Mice	RBC ($x10^6$ cells/μl)	Hb (g/dl)	Hct (%)	MCV (fl)	MCH (pg)	RDW (%)	Reticulocyte (%)	ROS (Mean FI)	PS cell (%)
WT	8.37±0.54	13.35±0.85	39.32±3.22	46.95±1.41	15.96±0.48	12.64±1.25	3.88±0.74	7.51±3.85	1.06±0.56
HT Hb E	7.95±0.36	12.24±0.56	35.69±1.58	44.93±0.84	15.40±0.35	14.47±0.79	4.65±0.86	3.28±0.43	0.87±0.29
BKO	5.34±0.63	7.06±0.61	22.86±2.05	43.05±2.95	13.32±1.09	23.17±2.53	29.01±5.76	170.1±24.6	3.35±0.75
DH	8.32±0.51	12.81±1.09	38.55±3.15	46.34±2.33	15.41±0.96	14.15±1.16	3.64±1.70	13.86±2.98	1.07±0.35
Rescued β/ Hb E	8.87+0.87	11.08±1.17	38.02±3.54	42.93±2.06	12.52±0.51	20.03±1.43	15.12±3.33	117.4±67.2	1.24±0.72

Abbreviations: Hb = hemoglobin, Hct = hematocrit, MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin, RDW = RBC distribution width, ROS = reactive oxygen species, PS = phosphatidylserine.

Table 2. Hematological parameters (mean±SD) of erythrocyte WT and β-thalassemia mice (Redrawn from Wannasuphaphol et al. [136]

5.2.1. Curcuminoids

Curcuminoids derived from *Curcuma longa* Linn. (turmeric) contained curcumin (diferuloylmethane), demethoxycurcumin (*p*,*p*′-dihydroxyldicinnamoylmethane), as well as *bis*-demethoxycurcumin (*p*-hydroxylcinnamoylmethane), which curcumin was found to be the most abundant and major active compound (Figure 7). Phenolic, methylene and β-diketo groups in the curcumin molecule participated in antioxidant, iron-chelating, free radical-scavenging and anti-lipid peroxidation activities.

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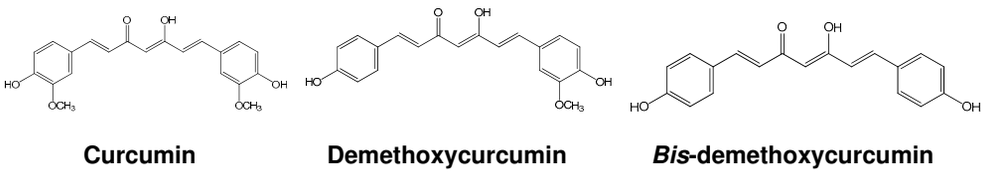


Figure 7. Chemical structures of curcumin, demethoxycurcumin and *bis*-demethoxycurcumin found in turmeric *Curcuma longa* Linn (Redrawn from http://www.molecular-cancer.com/18 September 2013)

Interestingly, curcumin can bind ferric and ferrous ions, in a concentration-dependent manner and with a molar ratio of 1:1, to form a red colored complex with a predominant peak at 500 nm. The curcumin itself chelates biological iron (e.g. plasma NTBI) and can work with DFP in lowering plasma NTBI levels more efficiently *in vitro* [139]. Curcuminoids effectively reduced levels of plasma NTBI, and liver weight index, non-heme iron, plasma and liver MDA concentrations, but increased the plasma GSH concentrations of iron-loaded BKO thalassemic mice [138]. Our results of the atomic absorption spectrometric and Perl's staining examinations demonstrated that curcumin was able to decrease the accumulation of heart iron and to depress heart rate variability of BKO mice with iron overload, suggesting the cardioprotective effect of curcumin [140]. Treatment of β-thalassemia HbE patients with curcuminoids (Thailand GPO product, 500 mg/day) for three months increased erythrocyte SOD as well as the GPx activities and GSH concentrations, and lowered levels of plasma NTBI and platelet factor-3 like activity [141, 142]. The same researcher group has been using a cocktail containing DFP, vitamin E, NAC and the curcuminoids for treatment of the thalassemia patients and expected that the treatment would improve their iron overload and oxidative stress more effectively (Kalpravidh and coworkers, personal communication). A recent study has reported that iron content of HDL-2 and HDL-3 from β-thalassemia/HbE patients was higher while the cholesterol content was lower than those levels found in the healthy subjects. Thalassemic HDL-2 and HDL-3 had increased levels of conjugated diene, lipid hydroperoxide and TBARS. The thalassemic HDL had lower peroxidase activity than the control HDL and could not protect against $CuSO_4$-induced oxidation of LDL [143].

5.2.2. Tea

Recently, the demand for green tea has increased due to current trends in human health concerns and preference. The main components found in green tea are polysaccharides, flavonoids, vitamins B, C, E, γ-aminobutyric acid, catechin compounds and fluoride. Among them, catechin compounds have been of focus for their strong antioxidant capacity. The pharmaceutical activities of the components have been studied. Tea (*Camellia sinensis*) is an excellent source of polyphenols, namely catechins, including (-)-epicatechin (EC), (-)-epicatechin 3-gallate (ECG), (-)-epigallocatechin (EGC), (-)-epigallocatechin 3-gallate (EGCG), (+)-catechin (C) and (-)-gallocatechin (GC). Among them, EGCG exerted the strongest antioxidant capacity and was found to be the most abundant, as well. It has been reported that catechins possess free radical scavenging abilities and iron chelating properties [144]. Green tea also showed a protective effect under various oxidative-related pathologic conditions.

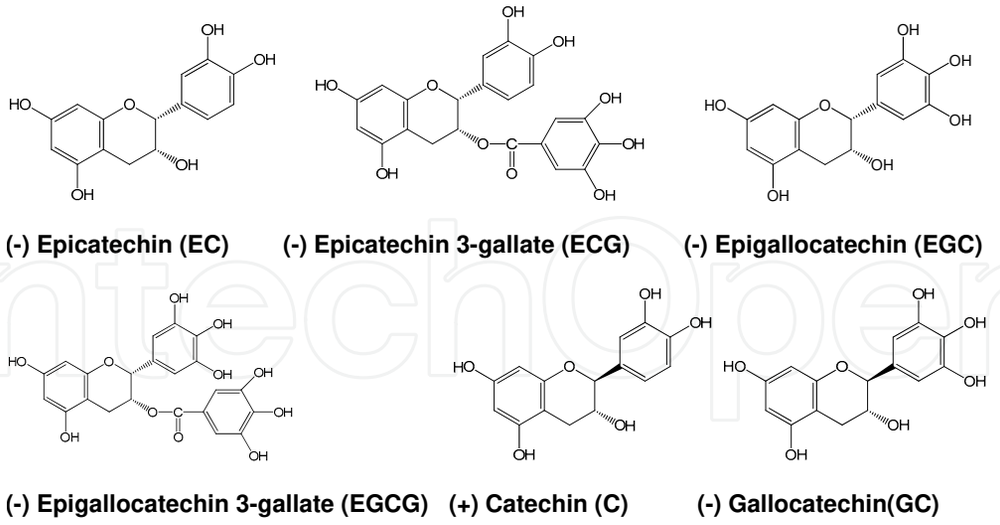


Figure 8. Chemical structures of catechins in tea (*Camellia sinensis*) (Redrawn from [145])

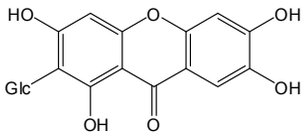
Drinking tea produced a 41 – 95% inhibition of dietary iron absorption in five β-thalassemia major and one β-thalassemia intermedia cases, which the iron absorption increased strikingly in the β-thalassemia intermedia cases, in references [146, 147]. Interestingly, Thai researchers have elucidated that green tea extract (GTE) and EGCG fraction were able to decrease iron (as NTBI) in plasma, eliminate plasma lipid-peroxidation product (as TBARS) and destroy the formation of erythrocyte ROS *in vitro* [144, 148] and in iron-loaded rats [149]. In addition, the GTE inhibited or delayed the deposition of hepatic iron in regularly iron-loaded BKO thalassemic mice effectively. This implies a prevention of iron-induced ROS generation and consequently liver damage and fibrosis by green tea consumption [150]. Our group has found that elevated levels of plasma NTBI and lipid peroxidation tended to be normalized in the BKO mice in response to oral therapy with GTE, while their plasma GSH concentrations were also increased by up to 2-times. The mice exhibited a decrease of the lipid peroxidation product and an improvement in the oxidant–antioxidant balance in erythrocytes. Importantly, GTE was effective in inhibiting hemolysis and thereby prolonged RBC half-life in the BKO mice (Sakaewan Ounjaijean PhD thesis. Chiang Mai University; 2011). Our current study has shown that the treatment of iron-loaded mouse hepatocytes and human hepatoma (HepG2) cells with GTE (0 – 100 mg/dl) and EGCG (0 – 200 μM) removed intracellular LIP and ROS efficiently, and relieved the mitochondrial membrane collapse, implying a hepatoprotective effect of green tea catechins in the hepatocytes with iron overload [151].

5.2.3. Mango (Mangifera indica L.)

Mangoes can be considered a good source of dietary antioxidants, such as ascorbic acid, carotenoids and phenolic compounds. *β*-carotene was found to be the most abundant carotenoid in several cultivars. The nutritional value of mango is that it is a source of vitamin C and provitamin A. Mangiferin (1,3,6,7-tetrahydroxyxanthone-2-glucopyranoside) (Figure 9) can interact with iron and other cations and also shows antioxidant activity by eliminating the superoxide radical *in vitro*, in which 100 μM of mangiferin was equivalent to the activity of 1

U/ml of SOD. This also revealed the pharmacologic effects modulating gene expression that were related to the inflammatory response [152]. Mangiferin xanthone modulates the expression of many genes critical for apoptosis regulation, viral replication, tumorigenesis, inflammation and autoimmune diseases, suggesting its possible value in the treatment of inflammatory diseases and/or cancer [153]. Vimang mango peel extract with a high mangiferin content acted as an antioxidant and complexed with Fe^{3+} efficiently, leading to protection of iron-induced oxidative liver damage and DNA fragmentation [154, 155]. Hydrolysable gallotannin present in mango kernels showed the inhibitory effects of bacterial growth, which was probably due to their iron-complexing properties [156].

Ripe and green Mahajanaka mango (www.nanagarden.com)



mangiferin

Figure 9. Fresh mango and chemical structure of mangiferin (Redrawn from [157])

Kaew mango peel extract can chelate both Fe^{3+} and Fe^{2+} to form the products with different predominant wavelengths, of which the binding was found to be dose-dependent and affinity-different. The green peel extract tended to exhibit stronger iron-binding abilities than the ripe peel extract and it is likely that the green peel might contain different kinds and amounts of phytochemical ingredients (Figure 10).

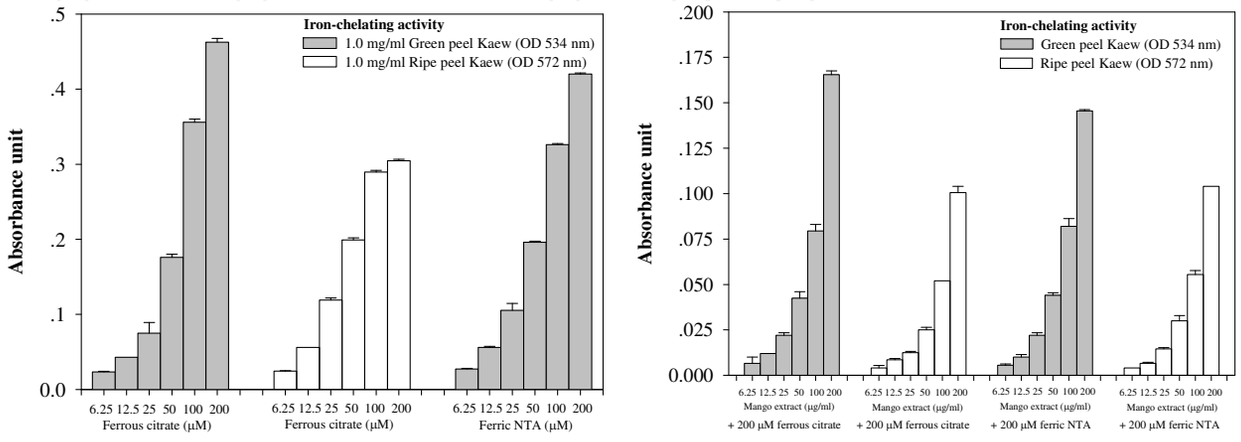


Figure 10. Iron-chelating activity of aqueous extract of Kaew mango peel (Srichairatanakool, S. unpublished data)

As shown in Table 2, the degree of antioxidant activity of the mango peel extracts were found to be Mahajanaka (ripe > green) > Chok-anan (green > ripe) > Namdocmai (green > ripe) > Kaew (ripe ~ green). The antioxidant activities were well correlated with their total phenolic contents and vitamin C concentrations. Incredibly, amounts of vitamin C in the extracts were lower than those of the total phenolic compounds. Mangiferin contents in the peel extracts were very low.

Mango/Status	Antioxidant activity (mg TEAC/g extract)	Total phenolics (mg GAE/g extract)	Vitamin C (mg/g extract)	Mangiferin (mg/g extract)
Mahajanaka/Ripe	225.6±4.4	107.6±9.6	5.49±0.23	1.49±0.25
Mahajanaka/Green	114.3±7.4	44.8±5.9	0.96±0.07	0.57±0.10
Chok-Anan/Ripe	120.1±5.4	46.0±7.0	0.75±0.08	4.62±0.37
Chok-Anan/Green	192.4±4.5	85.7±7.7	4.22±0.22	6.80±0.06
Namdocmai/Ripe	102.9±4.9	49.7±8.5	0.69±0.03	0.25±0.01
Namdocmai/Green	33.1±2.6	5.3±0.8	0.27±0.091	0.25±0.01
Kaew/Ripe	63.2±0.2	34.8±0.7	ND	0.02
Kaew/Green	65.3±0.1	55.9±0.3	ND	0.43

ND = not determined.

Table 3. Antioxidant activity, total phenolics, vitamin C and mangiferin contents (mean±SD) in aqueous extracts from ripe and green mango peels (Sricharatanakool, S. unpublished data)

5.2.4. Rice (Oryza sativa and Oryza glaberrima)

Rice is an economic cereal plant ±that is grown in many countries in Asian and Africa. Varieties of Asian rice include Thai rice (*O. sativa* cv. indica), Indonesian rice (*O. sativa* cv. javanica) and Japanese rice (*O. sativa* cv. japonica). Additionally, we have listed African rice (*O. glaberrima*). Regarding its nutritional values, rice grain contains carbohydrates (e.g. amylose and amylopectin) and rice bran is abundant in inositol, inositol hexaphosphate (or phytate), oil, ferulic acid, γ-oryzanol, phytosterol and tocotrienol. The ingredients in rice bran likely prevent carcinogenesis, hyperlipidemia, fatty liver, gallstone disease and heart diseases [158]. Red and purple rice bran possesses several fold higher hydrophilic and lipophilic anti-oxidative phenolics and flavonoids (predominantly cyanidine-3-glycoside) levels than freeze-dried blueberry and broccoli. Rice grass contains chlorophyll as a major ingredient and pheophytin (or pheo) as the second most abundant component (Figure 11). Pheophytin is synthesized in rice grass, spinach (*Spinacia oleracea*) leaves and *Michelia alba* leaves, but is not a degradation product of chlorophyll [159], and the compound reveals a level of antioxidant activity that is similar to that of ubiquinone [160].

Spinach pheophytin a (λ_{max} 409 nm) and pheophytin b (λ_{max} 435 nm) can chelate ferric ion and produce the Fe-pheophytin a complex (λ_{max} 393 nm) and Fe-pheophytin b complex (λ_{max} 421 nm) [161]. Germinating rice grain synthesizes iron-chelating compounds, deoxymugineic acid

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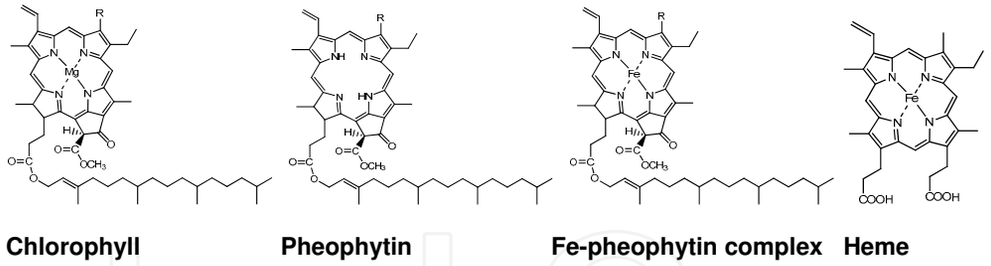

Figure 11. Chemical structures of chlorophyll, pheophytin, iron-pheophytin complex and heme [161]

and nicotinamine, and sequesters iron from the ground for their growth and development [162]. Interestingly, wheat grass (WG) juice which has been used as a general-purpose health tonic in Indian medicine and has shown at least a 25% reduction in the number of blood transfusions needed in β-thalassemia major patients [163] and also increased their blood hemoglobin levels [164]. Nonetheless, a contradictory report has shown that the oral administration of WG juice tablets for one year was not effective in reducing blood transfusions in transfusion-dependent β-thalassemia major patients [165].

Pimpilai and colleagues from Maejo University, Chiang Mai investigated the antioxidant activity of the Thai black rice grass (TBRG), Thai red rice grass (TRRG) and Thai fragrant rice grass (TFRG) extracts by using the TEAC colorimetric method and found that they exhibited antioxidant properties. The extracts dose dependently bound Fe^{3+} (ferric NTA) and Fe^{2+} (ferrous citrate) and formed the complex(s) with maximal absorption values at 393, 378 and 580 nm, respectively (personal communication). HPLC analysis showed different amounts of catechin derivatives as follows: 3.27 mg C, 4.51 mg EC and 5.19 mg EGCG in 1 g TBRG extract; 4.48 mg C, 6.22 mg EC and 5.23 mg EGCG in 1 g TRRG extract; and 5.89 mg C, 4.38 mg EC and 5.26 mg EGCG in 1 g TFRG extract. Currently, Pimpilai and Srichairatanakool have found that feeding rice grass (Sukhothai 1) extract (100 mg/kg body weight) along with a high iron diet to β-thalassemia mice for three months slightly increased their blood hemoglobin concentrations from 10.04±0.51 to 11.04±1.14 g/dl (unpublished data), while treatment with the extract (0, 50 and 100 μg/ml) decreased levels of liver MDA (153.6±44.7, 102.9±22.4 and 102.4±58.0 μg/mg tissue protein, respectively) and levels of labile iron (100±13, 120±1 and 125±5% fluorescent intensity, respectively) in iron-loaded HepG2 cell cultures. Therefore, the rice grass extracts would contain antioxidant compounds including chlorophyll, pheophytin and catechin derivatives, of which the two latter may play important roles in iron chelation and anti-lipid peroxidation to ameliorate oxidative tissue damage in the thalassemia cases with iron overload. Consequently, rice grass extracts need to be clinically studied in thalassemia patients in the near future.

6. Evaluation of oxidative stress and antioxidant status in thalassemias

Electron paramagnetic resonance (EPR) 'radical probe' was used to determine the total oxidative status in patients affected by thalassemia, and to evaluate new strategies of chelation,

new chelators, or the efficacy of antioxidant formulas [166, 167]. Raman spectroscopic technique has been developed for the monitoring of Raman hemoglobin bands to evaluate oxygenation capability, oxidative stress and deformities of thalassemic erythrocytes and to assess the responses to drug therapies [168]. Consistent with the study, in reference [169], serum PON activity and total antioxidant capacity were significantly lower in patients with the β-thalassemia trait patients, MDA and carotid artery intima-media thickness were significantly higher in β-thalassemia trait. The total antioxidant capacity, MDA, and CIMT levels were correlated with serum PON1 (r = 0.945, -0.900, 0.940 and -0.922 respectively). Additionally, serum total antioxidant capacity and MDA levels were well correlated (r = -0.979) [170].

An earlier study in 1986 showed that patients with Hb H diseases, including α-thalassemia 1 or α-thalassemia 2 and 21 with α-thalassemia 1/Hb Constant Spring, had increased activities of erythrocyte SOD, GPx, and CAT when compared with those of the controls. The α-thalassemia 1/Hb CS patients had higher SOD and GPx activities, but lower CAT activity than the patients with α-thalassemia 1/2 [171, 172]. One year later, a study of oxidative stress and the antioxidants in β-thalassemia/hemoglobin E patients in Thailand was conducted [173]. Significantly higher levels of urine N-acetyl-β-D-glycosaminidase, MDA and β_2-microglobulin along with aminoaciduria and proximal tubular abnormalities were found in pediatric α-thalassemia patients (Hb H disease and HbS/CS), and this was possibly due to increased oxidative stress [174]. A one-year treatment with DFP significantly decreased serum ferritin, NTBI, and MDA (p <0.05) of transfusion-independent β-thalassemia/HbE patients. Mean pulmonary arterial pressure and pulmonary vascular resistance were diminished significantly (p <0.05). All those parameters were still improved after subgroup analysis was done for the high ferritin group (>2500 ng/ml). The results imply that DFP therapy alone improved iron overload and oxidative stress and the compliance was positive [175]. Oxidative stress was increased in Thai HbE/β-thalassemia patients, as the blood GSH decreased, GSH/GSSG ratio reduced markedly, superoxide anion released from blood cells elevated highly, and γ-glutamylcysteine ligase activity was increased. Additionally, basal forearm blood flow was significantly increased whereas forearm vasodilatory response to reactive hyperemia was depressed [176].

When using ESR spectroscopic quantification of hemin, the serum hemin readily catalyzed free radical reactions and it would be a major pro-oxidant in the blood circulation of β-thalassemia Hb E patients [177]. A previous study showed a precipitous decrease in α-tocopherol and increased TBARS concentrations in both plasma and lipoproteins obtained by Thai β-thalassemia Hb E patients. Cholesteryl linoleate showed a reduction of 70% in LDL, while other cholesterol ester levels showed a lower reduction. A good correlation of NTBI and TBARS (p <0.01) in LDL strongly supported the contention that iron overload is responsible for initiating the lipid peroxidation in thalassemia patients [178]. The ESR results demonstrated a magnitude of increased lipid fluidity in thalassemic lipoproteins. Lipid fluidity at the LDL and HDL cores showed a good correlation with the oxidative stress markers and the α-tocopherol level, suggesting that the hydrophobic region of the thalassemic lipoprotein would be a target site for oxidative damage [179]. Gas chromatography/mass spectrometric (GS/MS) technique has been validated in quantifying ortho- and meta-tyrosine as biomarkers of protein

oxidative damage in plasma samples of β-thalassemia patients [180]. Pumala et al. have found that PON1 activity was significantly reduced in association with oxidative stress in the patients with β-thalassemia hemoglobin E, and significant correlations were observed between HDL-PON1 activity and oxidative stress markers (including plasma α-tocopherol and the ratio of cholesteryl linoleate to cholesteryl oleate in HDL, and a marked increased platelet-activating factor/acetylhydrolase (PAF-AH) activity [181]. The GC/MS-based assay showed that the level of urinary and plasma lipid peroxidation poroduct, F(2)-isoprostane in the thalassemic group was significantly increased [182]. An average antioxidant level in Thai thalassemia patients with the HbE trait (3.276±0.209 mM TEAC) was significantly decreased (p = 0.008) when compared to the healthy subjects (3.439±0.220 mM TEAC) [183]. It has been reported that Thai thalassemia major patients are associated with an alteration of CYP2E1 and CYP3A4 activities [184], but not CYP1A2 [185]. Anemia was not pronounced in the rescued mice (C57BL/6) with the Hb E transgene mimicing the human β-thalassemia/HbE phenotype; nonetheless, other hematologic parameters in their RBC include highly oxidative stress, no marked changes in PS and vesicles, and a shortened life span, which were abnormally similar to the BKO thalassemic RBC group [136].

7. Conclusions

Under iron overload, oxidative stress plays a major role in the pathophysiologic complications of thalassemia patients. Free extracellular toxic iron (e.g. NTBI and LPI) and intracellular redox iron (e.g. LIP and plasma membrane nonheme iron) that have been identified in thalassemic blood and tissues are responsible for the generation of oxidative stress by catalyzing a formation of oxygen radicals over the antioxidant capacity of the cells. Consequently, there is a rationale to support iron chelation therapy for the elimination of the free-iron species and to promote the free-radical scavenging activity of the antioxidants. Not only synthetic (vitamin C, vitamin E and NAC) but natural (e.g. polyphenolics, flavonoids and fish oils) antioxidants are also capable of ameliorating such increased levels of oxidative stress. Taken together with an effective iron chelator, antioxidants may provide a substantial improvement in hemolytic anemia cases, and particularly in thalassemia patients. Most importantly, natural antioxidants are ubiquitous and very cheap whereas antioxidant supplements are free from the side effects commonly encountered in iron chelation and chemotherapeutic treatments.

Abbreviations

ACE-II = angiotensin-converting enzyme II
AD4 = *N*-acetylcysteine amide
δ-ALA = delta-aminolevulinic acid
ALP = alkaline phosphatase
ALT = alanine aminotransferase

ApoB = apoprotein B

ApoE = apoprotein E

AST = aspartate aminotransferase

BKO = beta-knockout

CAT = catalase

CVD = cardiovascular disease

COX = cyclooxygenase

DFO = desferrioxamine

DFP = deferiprone

DFX = deferasirox

eNOS = endothelial nitric oxide synthase

Epo = erythropoietin

EPR = electron paramagnetic resonance

FI = fluorescent intensity

FPP = fermented papaya preparation

GA = gallic acid

GC/MS = gas chromatography/mass spectrometry

GGT = γ-glutamyl transferase

GPx = glutathione peroxidase

GTE = green tea extract

GSH = reduced glutathione

iNOS = inducible nitric oxide synthase

iv = intravenously

im = intramuscularly

IRP = iron-regulatory proteins

LDL = low-density lipoprotein

LIP = labile iron pool

LPI = labile plasma iron

MRI = magnetic resonance imaging

NAC = *N*-acetylcysteine

NAD(P)H = nicotinamide adenine dinucletide (reduced)

nNOS = neuronal nitric oxide synthase

NOX = NAD(P)H oxidase

NTBI = non-transferrin bound iron

PAF-AH = platelet-activating factor/acetylhydrolase

PBMC = peripheral blood mononuclear cells

PMA = phorbol myristyl acetate
PON1 = paraoxonase 1
PMN = polymorphonuclear cells
RBC = red blood cells
RES = reticuloendothelial system
ROS = reactive oxygen species
sc = subcutaneously
SI = serum iron
SOD = superoxide dismutase
TBARS = thiobarbituric acid reactive substances
TBI = transferrin-bound iron
TBRG = Thai black rice grass
TFRG = Thai fragrant rice grass
TRRG = Thai red rice grass
TIBC = total iron-binding capacity
WG = wheat grass
XO = xanthine oxidase

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