Chapter 5

Diet-Related Thalassemia Associated with Iron Overload

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Additional information is available at the end of the chapter

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Abstract

Thalassemia is an inherited disease caused by the genetic disorder of α- and β-globin genes, resulting in ineffective erythropoiesis and chronic anemia. Transfusion-dependent β-thalassemia patients require red cell transfusion to maintain their blood hemoglobin level in the normal range, whereas non-transfusion-dependent thalassemia patients increase duodenal absorption of dietary iron in an attempt to accelerate erythropoiesis. These changes give rise to iron overload, oxidative stress, organ dysfunction, and other complications. Effective iron chelators are necessary to achieve negative iron balance and to relieve such complications associated with iron overload. Some pharmaceuticals such as hydroxyurea, N-acetylcysteine, ascorbic acid, vitamin E, and glutathione are also given to thalassemia patients in order to overcome oxidative cell and tissue damage and to generate a better quality of life. Interestingly, functional natural products (such as mango, tea, caffeine, and curcumin), vegetables, and cereal (e.g., rice) are helpful for their health-providing properties by supplementing the endogenous antioxidant defensive power in the body. Natural products exhibit many pharmacological activities, but they are safer if used in the traditional manner.

Keywords: thalassemia, personalized medicine, antioxidant, green tea, functional fruits, iron

1. Introduction

Thailand is one of the countries located in Southeast Asia (SEA) with an ongoing thalassemia endemic and has been affected by this inherited disease for a long time. In 2012, we had an official meeting for reviewing progression in the field to develop a good clinical practice guideline (CPG) for thalassemia management in Thailand.

2. Etiology of thalassemia

Thalassemia is an inherited autosomal recessive disorder of hemoglobin molecules (ineffective erythropoiesis) that is characterized by an imbalanced α- and β-globin chain synthesis. The accumulation of unbound α-globin chains in erythroid cells is the major cause of pathology in β-thalassemia. Stimulation of γ-globin chain synthesis can relieve disease severity because it combines with the α-globin chain to form a fetal hemoglobin (Hb F). The disease occurs prevalently from Southeast Asia to the Mediterranean.

2.1. α-Thalassemia

α-Thalassemia is due to an impaired production of α-globin chains from 1, 2, 3, or all 4 of the α-globin genes, leading to a relative excess of β-globin chains. The severity of the disease is based on how many genes are affected. Four clinical conditions of increased severity are recognized: two carrier states, α^{+}-thalassemia caused by the deletion or dysfunction of one of the four α-globin genes, and α^{0}-thalassemia resulting from deletion or dysfunction of two α-globin genes in *cis*. The two clinically relevant forms are Hb Bart's hydrops fetalis syndrome and Hb H disease. Patients with Hb Bart's hydrops fetalis syndrome (homozygous α-thalassemia) have nonfunctioning α-globin genes (genotype α-thal 1/α-thal 1 or − −/− −) and mostly die before birth. Mothers usually suffer hypertension, edema, and toxic pregnancy. Hb H disease patients carry only one functioning α-globin gene (genotype α-thal 1/α-thal 2 or − −/− α, and α-thal 1/Hb Constant Spring (CS) or −/α^{CS}α) and mostly suffer mild-to-severe anemia, jaundice, febrile, and splenomegaly and hepatomegaly. α-Thalassemia is prevalent in tropical and subtropical regions similar to other common globin gene disorders such as β-thalassemia and sickle cell anemia where malaria was and still is an epidemic. As a consequence of massive population migrations, α-thalassemia has become a relatively common clinical problem in North America, Europe, and Australia [1–3].

In northeast Thailand, thalassemia patients suffered with Hb H disease mostly due to the interaction of α-thalassemia 1 (SEA type) with the Hb CS, the deletion of three α-globin genes with the SEA type α-thalassemia 1 and the 3.7- or 4.2-kb deletion of α-thalassemia 2, and the interaction of the SEA α-thalassemia 1 with the Hb Pakse [4]. In Cambodia, α-globin gene mutation was mostly caused by the α-(3.7) (rightward) deletion (frequency 0.098–0.255), α-thal-1 (− −(SEA)) (frequency 0.008–0.011), and α-thal-2 [-alpha(4.2) (leftward deletion)] (frequency 0.003–0.008) [5].

2.2. β-Thalassemia

Human β-thalassemia is characterized by the deficient production of the β-globin chains of adult hemoglobin (Hb A), typically due to mutations of the β-globin gene. Over 200 mutations have been identified in this gene, and the type of mutation can influence the severity of the disease. There are three main types of β-thalassemia, listed in order of decreasing severity: homozygous β-thalassemia major (TM) (genotype β^{0}/β^{0}) caused by mutations in both alleles, β-thalassemia intermedia (TI) (genotype β^{0}/β^{+}, β^{+}/β^{+}, and β^{+}/β^{E}) caused by diverse mutations, and heterozygous β-thalassemia minor caused by single mutation, including hereditary persistent fetal hemoglobin (HPFH). TI patients usually become mildly anemic (baseline Hb level 7–10 g/dl) and have

widely varying severity. Some patients require blood transfusion and chelation to promote their growth in childhood and prevent bone deformities in adults and sometimes get splenectomy due to hypersplenism and mechanical encumbrance. Enhancing Hb F synthesis is useful in some patients, and anti-oxidative compounds were found not to improve blood Hb levels. Stem cell transplantation and gene therapy are possible in well-developed countries but limited in developing countries and in some severe cases. Many complications such as pulmonary hypertension, thrombosis, hypercoagulability, pseudoxanthoma elasticum, and osteoporosis are reported in TI patients and can affect their treatment [6].

β-Thalassemia hemoglobin E (Hb E) (genotype β^0/β^E or β^+/β^E) is most prevalent in SEA countries including Thailand where the carrier frequency is around 50%. The interaction of thalassemia Hb E and β-thalassemia results in a clinical spectrum ranging from a condition indistinguishable from TM to a mild form of TI. Three categories can be identified depending on symptoms as followed: asymptomatic (normal Hb level), mild (baseline Hb level <9.0 g/dl), moderate (baseline Hb level 7–9 g/dl), and severe (baseline Hb level <7.0 g/dl). In transgenic mice, homozygous beta-knockout (BKO) thalassemia shows many clinical features of red blood cells (RBC) indices, in particular mild anemia similar to human TI. The abnormalities include decreased blood Hb concentration, hematocrit (Hct), numbers and osmotic fragility of RBC, and the increase of reticulocyte count. Additionally, Perl's staining and colorimetric assays shows deposition of iron in the spleen, liver, and kidneys but not in the heart [7].

3. Anemia in thalassemia

The accumulation of excess unbound α-globin chains in erythroid cells of β-thalassemia patients can result in RBC hemolysis and anemia; nevertheless, stimulation of γ-globin gene to produce γ-globin chain which can combine with the α-globin to form Hb F is a therapeutic approach. Like cell apoptosis, eryptosis is a programmed cell death or suicidal death of erythrocytes which is characterized by shrinkage, membrane bleb, activation of proteases (e.g., caspase and calpain) after oxidative stress, and phosphatidylserine (PS) exposure at the outer plasma membrane leaflet of the affected RBC. Eryptosis can be triggered by osmotic shock, energy depletion, hyperthermia, curcumin, ceramide, prostaglandin E_2, platelet-activating factor, valinomycin, amyloid peptide, hemolysin, chlorpromazine, cyclosporine, paclitaxel, stressors-induced injury, and iron-induced oxidative stress. In contrast, it is inhibited by erythropoietin (EPO), catecholamines, and nitric oxide (NO). Eryptosis is probably a useful mechanism to get rid of defective RBC and infectious agents. Nonetheless, excessive eryptosis found in iron deficiency, intoxication of metals (such as Al, Cu, Pb, and Hg), xenobiotics, β-thalassemia, sickle cell disease (SCD), glucose-6-phosphate dehydrogenase (G6PD) deficiency, hereditary spherocytosis, paroxysmal nocturnal hemoglobinuria, myelodysplastic syndrome (MDS), phosphate depletion, sepsis, hemolytic uremic syndrome, renal insufficiency, diabetes, pathogenic infection (e.g., malaria, mycoplasma, and hemolysin-producing bacteria), and Wilson's disease can result in short lifespan and microvesicles of the RBC, consequently leading to anemia and impaired microcirculation [8–10]. Synthetic compounds and natural products of interests need to be investigated to elucidate their therapeutic potential of inhibitors of excessive eryptosis in β-thalassemia with chronic anemia.

4. Iron overload in thalassemia

4.1. Pathophysiology and complications

Iron overload in thalassemia is assessed with an increase of plasma iron and transferrin saturation, the presence of redox iron as non-transferrin-bound iron (NTBI) and labile plasma iron (LPI), and a high deposition of tissue iron in the forms of hemosiderin, ferritin, and labile iron pools (LIP). Excessive iron accumulation in the vital organs is the cause of liver diseases (e.g., hepatitis, hepatic fibrosis, and hepatocellular carcinoma), cardiomyopathies (e.g., cardiac arrhythmia and heart failure), and endocrinopathies (e.g., diabetes, growth retardation, defective puberty, hypopituitarism, hypogonadism, and hypoparathyroidism) [11, 12]. Iron overload can be caused by an increase of dietary iron absorption due to chronic anemia and by multiple blood transfusions to maintain normal blood Hb level. Under incomplete or partial synthesis of β-chains of Hb in β-thalassemia patients, the remaining excessive α-globin chains are unstable and eventually precipitate, causing RBC membrane damage [13]. The affected RBCs are prematurely hemolyzed in the bone marrow and spleen, resulting in increased RBC turnover, ineffective erythropoiesis, and severe anemia, so patients require regular blood transfusions to prevent the anemia and ischemia. Though thalassemia patients do not receive transfusions, abnormal iron absorption produces an increase in the body iron burden evaluated at 2–5 g per year [14]. Regular blood transfusions (420 ml/unit of donor blood equivalent to 200 mg of iron) lead to double this iron accumulation. Consequently, iron accumulation introduces progressive damage in the liver, heart, and in endocrine glands. Circulating NTBI as well as LPI is detected whenever the capacity of transferrin to incorporate iron derived from either gastrointestinal tract or reticuloendothelial (RE) cells becomes a limiting factor. Both forms of toxic iron appear primarily in transfused patients where the total iron-binding capacity (TIBC) has been surpassed [15]. Pathologically, the NTBI fraction seems to be translocated across cell membrane irregularly, while the LPI is redox active and susceptible to chelation [16].

4.2. Redox iron catalysis

In enzymatic reactions as shown in **Figure 1**, superoxide ($O_2^{-\bullet}$) which is one of the reactive oxygen species (ROS) is normally produced by NADH:ubiquinone oxidoreductase catalysis at the complex I (I) in oxidative phosphorylation and will be converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) catalysis (II). Hydrogen peroxide (H_2O_2) which is another ROS is produced by xanthine oxidase (XO) catalysis of hypoxanthine to xanthine (III) and xanthine to uric acid (IV) in purine catabolic pathway. Finally, hydrogen peroxide will be degraded or detoxified by peroxidase (POD) and catalase (CAT) to water and oxygen (V).

In Haber-Weiss/Fenton nonenzymatic reactions, iron can participate in the oxidation-reduction process known to generate ROS including hydrogen peroxide reacts to form hydroxyl radical ($OH^{\bullet}$) and hydroxide anion (OH^-) [17] (**Figure 2**).

ROS can induce cell death through initiating a series of chemical reactions with many significant biomolecules, resulting in DNA oxidation, protein damage, and membrane lipids peroxidation [18, 19]. Among these ROS, hydroxyl radicals might be the most harmful to lipid and

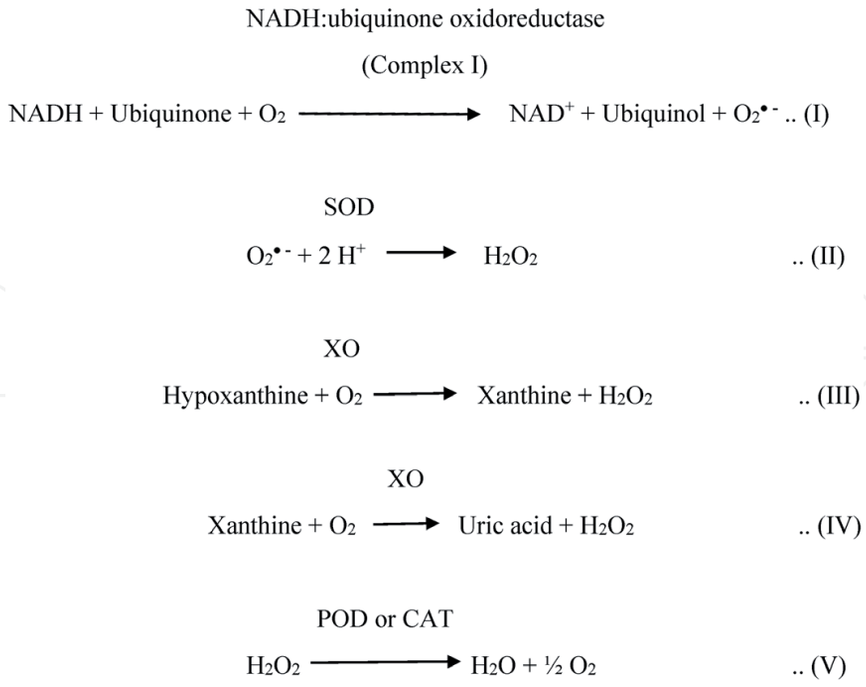

Figure 1. Enzymatic production of ROS.

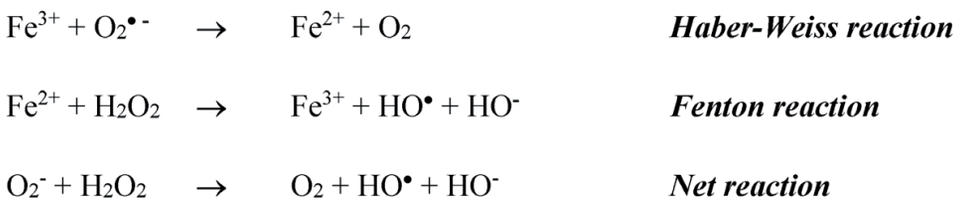

Figure 2. Iron-catalyzed redox reactions of biological importance.

protein membrane components. The •OH-induced membrane damage can be related directly to a membrane-associated Fenton reagent [20]. Oxidative cell damage has been attributed to the emergence of excessive levels of LPI that promote the production of ROS exceeding the cellular defensive capacity [21]. Cellular LIP is a source of chelatable and redox-active iron, which is transitory and serves as a crossroad of cell 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 (IRPs) and its feedback regulated by an IRP-dependent expression of iron import and storage. LIP is influenced by a range of biochemical reactions that are capable of overriding the IRP regulatory loops. Excess labile iron can react with unsaturated lipids [22]. Such redox reactions lead to the damage of cells, tissues, and organs as demonstrated as the iron overload associated with β-thalassemia.

4.3. Tissue iron deposition

The spleen contains macrophages that digests hemoglobin and stores the resulting iron in ferritin. The number of blood transfusions in β-TM patients correlates with their splenic

hemosiderosis and weight [23]. Hemosiderin deposition was found to be greater in the iron-overloaded livers than in the iron-overloaded spleens. Ferritin and hemosiderin increased in hepatocytes and splenic RE cells [24]. Splenectomy is one of the clinical complications of hypertransfused TM patients to reduce hyperactivity of RE macrophage; nevertheless, it may increase the iron overload.

The liver is one of the main storage organs for iron. Iron overload is considered when the ferritin level consistently exceeds 1,000 ng/ml (normal range 20–200 ng/ml). Excess free radicals can cause progressive tissue injury and eventually cirrhosis or hepatocellular carcinoma in iron overload patients whose iron is sequestrated predominantly in ferritin or hemosiderin [25]. When plasma transferrin becomes highly saturated, NTBI is detectable and rapidly transported across the hepatocyte membrane via a specific pathway. Likely, ferroportin 1 is the only protein that mediates the transport of iron out of hepatocytes and is then oxidized by ceruloplasmin and bound to transferrin [26]. Iron deposition affects hepatic parenchymal cells (hepatocytes and bile duct cells) and mesenchymal cells (endothelial cells, macrophage, and Kupffer cells) and often distributes differently from one area to another [27].

The heart is one of the most mitochondrial-rich tissues in the body, making the iron of particular importance to cardiac function. Iron as iron-sulfur cluster and cytochromes plays a key role for oxidative phosphorylation and superoxide production in the mitochondria. Iron deposition in the heart cells can lead to cellular oxidative stress and damage and an alteration of myocardial function. Heart failure is the leading cause of death among hemosiderosis β-thalassemia patients, of whom around 60% die from cardiac failure. Harmful effects of iron overload on the heart of TM patients can be monitored efficiently by using noninvasive techniques as described below, whereas invasive techniques such as Perl's stained in biopsied heart tissue are rather impossible. Treatment with effective iron chelators can protect these patients from iron-loaded cardiomyopathy [28, 29].

Bone marrow iron deposition (186 μg/g wet weight) increases in proportion to the total body iron store in dietary iron overload of African Bantu people and Caucasian idiopathic hemochromatosis patients [30]. MDS patients who are a heterogeneous group of clonal hematopoietic stem cell malignancies show bone marrow hemosiderosis and may develop systemic iron overload.

Though hematological care is improved in homozygous transfusion-dependent β-thalassemia (TDT) patients, multi-endocrine dysfunction is still a common complication. Thyroid dysfunction is defined as overt hypothyroidism, subclinical hypothyroidism, and an exaggerated thyroid-stimulating hormone response was reported in β-thalassemia patients [31]. Possibly, growth retardation, secondary hypogonadotropic hypogonadism and hypothyroidism are originated from pituitary damage primarily caused by iron overload and oxidative stress [32, 33]. Approximately half of patients' pituitary gland dysfunction associated with iron overload is irreversible [34].

The redox irons in TDT patients with TM and TE are catalytically harmful to adrenal glands and can cause adrenal insufficiency [35]. Though all TM patients were nondiabetic, some of them decreased in the oral glucose tolerance test. They showed normal response of cortisol to insulin and adrenocorticotropic hormone stimulation. Moreover, the β-cell pancreatic function and adrenal cortical function were depressed in the severely iron-loaded. Recently, Koonyosying and colleagues have demonstrated green tea extract could reduce cellular the

levels of iron and ROS and increase insulin secretion in concentration-dependent manner in iron-loaded pancreatic cell line (RINm5F), indicating the amelioration of oxidative stress and endocrinal improvement of pancreatic β-cells [36]. They also found that eltrombopag, which is a thrombopoietin receptor agonist and potent metal ion-chelating agent, efficiently decreased cellular levels of iron and ROS from cultured HuH7, H9C2, and RINm5F cells and restored insulin secretion from iron-loaded RINm5F cells [37].

4.4. Assessment of tissue iron content

Serum ferritin level has been used as a surrogate biochemical marker to correlate closely with liver iron concentration for a long time and would be a valuable alternative to assess visceral iron overload in heavily iron-loaded TM patients [38]. Sophisticated noninvasive magnetic resonance imaging, magnetic iron detector susceptometry, superconducting quantum interference device, and nuclear resonance scattering techniques can also be used to assess iron status in tissues. Alternatively, invasive tissue biopsied needle aspiration associated with ferrozine colorimetry or graphite-furnace atomic absorption spectrometry is routinely quantitated for nonheme iron in tissues (e.g., myocardium, liver, pancreas, adrenal glands, anterior pituitary gland, and skin) [39–41]. These methods are all valuable when evaluating iron load in the tissues and monitoring the response of different organs to chelation therapy.

5. Thrombotic events in thalassemia

Heart failure and arrhythmia are the main causes of death in TM patients with cardiac siderosis, pulmonary hypertension, and thrombosis and also the major cardiovascular complications in TI patients possibly due to pro-atherogenic biochemical factors (e.g., iron status and lipid profile) [42, 43]. Hypercoagulable pulmonary microthromboembolism in Thai pediatric TE patient was previously investigated [44]. After splenectomy TI patients mostly had thrombosis, thrombocytosis, and lower levels of anticoagulation inhibitors (e.g., protein S, protein C, and antithrombin III) [45]. Splenectomy promotes portal vein thrombosis in TM patients [46]. Ineffective erythropoiesis, chronic anemia, iron overload, and polycythemia by erythrocytosis and thrombosis are coincidently occurring in β-thalassemia patients. Signs of cerebrovascular accident (brain ischemia, hemorrhage, and infarct) and heart disease (congestive heart failure and atrial fibrillation) were described in chronically hypercoagulable thromboembolic thalassemia patients, so anticoagulant and/or antiplatelet therapy is recommended. Hypoxia and iron overload are the two major mechanisms of ROS overproduction [47]. The levels of plasma hemostatic and thrombotic markers were significantly higher in splenectomized TE patients than non-splenectomized ones, implying splenectomy increases platelet hyperactivity, blood hypercoagulability, and risk of thrombosis. ROS-induced activation of vascular endothelial cells can cause vasculitis and thrombosis, showing increased levels of many soluble adhesion molecules and von Willebrand factor (vWF) in thalassemia blood [48]. Procoagulant activity of circulating RBC microvesicles or microparticles (MPs) may contribute to thrombotic events in thalassemia hypercoagulability [49]. Carotid artery thrombus is usually associated with severe

cardiovascular diseases (CVD), iron deficiency anemia, and thrombocytosis. Thromboembolic complications are documented in thalassemia patients, possibly due to aggregation of abnormal RBC and high amounts of RBC membrane-derived MPs [50]. Antioxidant treatment of β-thalassemia HbE patients can improve oxidative stress and hypercoagulable state [51]. Iron overload, in particular NTBI level, would be one of the risk factors in pulmonary thrombosis and hypertension in splenectomized non-transfusion-dependent thalassemia (NTDT) patients [52]. Iron chelators are useful and effective in the amelioration of iron overload and oxidative stress in thalassemia mice, possibly in the prevention of pulmonary thrombosis [53]. Nitric oxide ($NO^{\bullet}$) synthesized from L-arginine by catalysis of nitric oxide synthase (NOS) species is a free-radical, physiologic vasodilator, and potent inhibitor of platelet function. Excessive iron-liberated heme degradation contributes to hypercoagulability [54]. Low arginine bioavailability in β-thalassemia patients can cause pulmonary hypertension and cardiopulmonary dysfunctions [55]. Splenectomy, thrombocytosis, RBC, and platelet MPs may be residual hypercoagulable/thrombotic risks in TDT patients [56, 57]. Liver inflammation and cirrhosis can involve in hypercoagulability, thrombosis, and reduced fibrinolysis [58, 59].

6. Treatment and implements

Strategy and approach have been suggested for the treatment and support of thalassemia patients to have better quality of life and well-being [60]. These approaches include occasional/regular blood transfusions, iron chelation therapy, antioxidant supplement, Hb F switching agents, anti-allergic drugs, antibiotics (such as antibacterial, antiviral, antifungal, and antimalarial drugs), splenectomy (in the past), dental care, and hemopoietic stem cell transplantation.

6.1. Iron chelation therapy

Iron chelation therapy aims to prevent the accumulation of toxic iron and eliminate the excess iron in TDT patients. Effective chelation and good management of the patients have been correlated with a decline in early deaths and complications [61]. Reduction of plasma and cellular chelatable iron such as NTBI, LPI, and LIP is a slow process and requires aggressive chelation therapy. The chelation will maintain the iron balance at safe levels to prevent high iron accumulation and oxidative tissue injury. Such non-iron and iron-overloaded models as RBC, cell cultures (e.g., hepatocytes, HepG2 cells, and cardiomyocytes), animals (e.g., mice, gerbils, rats, and transgenic BKO mice), and even human thalassemia patients are experimentally investigated and clinically tested to assess the safety and efficacy of iron chelators. At present, three standard iron chelators including desferrioxamine (DFO), deferiprone (DFP), and deferasirox (DFX) are widely used for the treatment of β-thalassemia patients with iron overload to prevent oxidative stress-induced organ dysfunctions and such complications (**Figure 3**). Combined DFO/DFP and DFP/DFX treatments can reverse endocrine complications by improving glucose intolerance and gonadal dysfunction in TDT patients [62].

Under continuous chelation therapy, many TDT patients with moderate-to-severe pituitary iron overload had normal volume and function of the pituitary gland, representing a potential therapeutic window, while some hypogonadal patients preserved their pituitary volumes

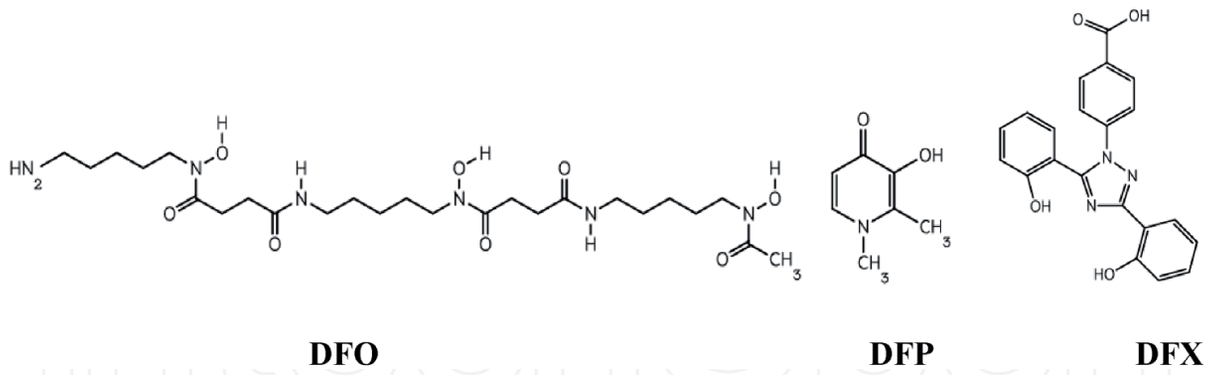

Figure 3. Chemical structures of DFO, DFP, and DFX.

and functions. Thai clinicians have reported that DFO chelation therapy for 1.5 years largely decreased serum ferritin level and improved secretion of prolactin (PRL) and growth hormone (GH) but not other pituitary hormones [63].

6.2. Supplementation of antioxidants

Compounds such as vitamins A, C, E, β-carotene, reduced glutathione (GSH), and *N*-acetylcysteine (NAC) and enzymes such as SOD, CAT, glutathione peroxidase (GPx), and glutathione reductase (GR) can remove free radicals by enzymatic and nonenzymatic antioxidant systems in the body (**Figure 4**). Since β-thalassemia patients have a higher oxidative stress level than normal people, effective antioxidants would be a complementary treatment of choice in these patients. Ideas for using drug antioxidants to eliminate oxidative tissue damage and empower antioxidant systems in thalassemia patients have been applicable for a long time [64]. Commercially available compounds included vitamin C, vitamin E, NAC, coenzyme Q_{10}, and hydroxyurea (HU) which were used for the treatment, with vitamin E being the most popular [65–79]. Importantly, treatment with vitamin E significantly lowered the levels of plasma lipid peroxidation products and adenosine diphosphate (ADP)-challenged platelet activity in non-splenectomized and splenectomized HbE/β-thalassemia patients [80]. Regarding other anti-oxidative natural products, silymarin restored glutathione level in thalassemia patients [81]. Fermented papaya preparation (FPP) increased glutathione levels in blood cells and platelets and decreased membrane lipid peroxidation products in β-thalassemia patients [82]. Treatment with a cocktail of DFP, NAC, vitamin E, and curcumin for 1 year improved antioxidant capacity in HbE/β-thalassemia patients [80, 83]. The levels of serum vitamins A and E, Zn, Se, and Cu were lower in young thalassemia patients than in controls, whereas serum ferritin and iron levels were inversely correlated with

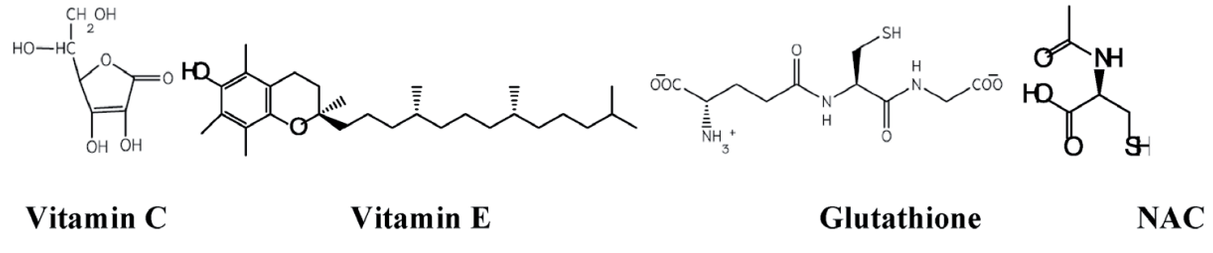

Figure 4. Structures of antioxidants.

serum retinol and selenium levels ($p < 0.05$). Interestingly, vitamin E and polyphenols can abolish increased oxidative stress in thalassemia patients; if given along with iron chelators, then they may provide a substantial improvement in chronic anemia and complications [84].

6.3. Vitamin C

Ascorbic acid or vitamin C is a simple water-soluble vitamin which cannot be enzymatically synthesized in the human body. The substance normally functions as a cofactor of proline and lysine hydroxylase in collagen synthesis. The levels of leukocyte and urinary AA are decreased in idiopathic hemochromatosis patients, TDT patients, and Bantu people [85]. Platelet vitamin C level is lower in thalassemia patients with iron overload than normal people [86]. When TM patients are treated with vitamin C, their levels of serum iron, transferrin saturation, and ferritin are increased [87]; possibly vitamin C would be involved in the mobilization of storage iron from tissues and increase oxidative damage in the patients. However, vitamin C plus vitamin E supplementation for β-thalassemia patients has benefits more than vitamin E alone in promoting their antioxidant activity [66].

6.4. Vitamin E

Vitamin E (α-tocopherol) is considered to be the most important lipid-soluble exogenous antioxidant in humans. Low serum level of vitamin E is found in homozygous TM and TE patients. Oral administration of high doses of vitamin E effectively decreased plasma lipid peroxidation in β-thalassemia patients and prolonged RBC survival in some patients [71, 88]. A therapeutic trial with vitamin E was carried out in TM and TI patients with 750–1000 IU/day for an average period of 16 months. The treated patients showed fourfold increase in both serum and RBC vitamin E levels and a reduced level of malonyldialdehyde (MDA) when compared with the untreated group [89]. Daily vitamin E supplementation for 3 months significantly increased plasma α-tocopherol levels and reduced plasma oxidant levels in splenectomized TE patients [80].

6.5. Glutathione

Glutathione (γ-glutamylcysteinylglycine) is a tripeptide synthesized by the catalysis of γ-glutamyl cysteine synthetase and glutathione synthetase in cells and indicated as a very important endogenous free-radical scavenger due to the presence of cysteine sulfhydryl group in the molecule. In addition, GR, GPx, and glutathione-*S*-transferase (GST) work as antioxidants to get rid of harmful free radicals mostly in the cells. Physiologically, GR together with reduced nicotinamide adenine dinucleotide phosphate functions to recycle oxidized glutathione (GSSG) back to GSH to scavenge ROS, and GPx converts hydrogen peroxide to water and oxygen. GSH is approximately 80% present in the liver. GR activity was slightly decreased in TDT patients, whereas GPx activity was not different when compared with healthy persons [90]. Blood GSH levels of α-, β-, and HbE/β-thalassemia patients with iron overload were significantly lower than those of the healthy controls [91–95]. Importantly, treatment with flavonoid silymarin restored a decreased GSH content in T cells

of β-thalassemia major patients [81]. Though endogenous GSH content is unable to be filled up with direct consumption due to digestive peptidase activity, oral administration of some antioxidants such as vitamin E (10 mg/kg/day), commercially available FFP, silymarin tablet (140 mg three times a day), HU (10–20 mg/kg/day), NAC (2,400 mg/day), and curcumin (500 mg/day)/vitamin E cocktail can increase/restore intracellular GSH content in thalassemia patients instead.

6.6. Hydroxyurea

HU (alternatively hydroxycarbamide) is a drug of choice used for enhancing γ-globin gene expression and modifying γ-globin chain production, as a consequence of Hb F production in SCD and β-thalassemia patients. In controversy, the compound is toxic and suspected to the pathogenesis of colonic ulcerative [96]. Indeed, HU effectively increases Hb F production in patients with SCD, SCD with α-thalassemia, and TI and results in a decrease in the number of blood transfusions required [97–99]. A current clinical study in TI patients has shown HU decreased serum ferritin (50 vs. 33%), LIP (20 vs. 13%), apoptotic event (62 vs. 15%), and ROS (60 vs. 50%) levels and increased GSH level (66 vs. 25%) in the responders compared to the nonresponders [100]. In addition to the increase in Hb F synthesis, treatment with HU (30 mg/day) in β-thalassemia patients with Hb E for 3 months decreased SOD activity and MDA concentration of the RBC, probably due to inhibition of cytosolic superoxide radical and membrane lipid peroxidation [101, 102].

6.7. *N*-acetylcysteine (NAC)

NAC, an anti-oxidative thiol-containing compound, is able to trap ROS and reactive nitrogen species (RNS) and therefore protect cells from such free-radical-mediated damage. After crossing the cell membrane, the compound will be hydrolyzed to cysteine used for the synthesis of GSH. Importantly, NAC can protect the RBC of SCD patients and of normal subjects from oxidative stress condition [65, 103]. In vitro treatment of blood cells including RBC, platelets (PLT), and polymorphonuclear leukocytes of β-thalassemia patients with *N*-acetylcysteine amide increased GSH content and reduced ROS level in these cells, possibly resulting in a significant reduction in the sensitivity of thalassemia RBC to hemolysis and phagocytosis by macrophages [65]. They also showed that the intraperitoneal injection of AD4 to β-thalassemia mice (150 mg/kg) significantly reduced all parameters of oxidative stress. One β-thalassemia with hemoglobin sickle (Hb S) who received NAC (2400 mg/day) for 6 weeks showed an increase in whole-blood GSH levels and a decrease in the RBC membrane PS exposure [104]. Consistently, TDT patients who received NAC (10 mg/kg/day) for 3 months showed a decrease in total oxidative stress and total oxidative stress index and an increase in total antioxidant capacity and blood Hb level [105]. Our group has reported that treatment of β-thalassemia HbE with a cocktail of DFP, NAC, and either vitamin E or cucumin for 12 months significantly decreased levels of iron overload (e.g., NTBI, LPI, erythrocyte membrane nonheme iron) and oxidative stress (e.g., MDA and erythrocyte ROS) parameters and increased levels of blood Hb and antioxidant indicators (e.g., CAT, SOD, and GSH), suggesting an effective antioxidant property [51].

7. Supplementation of functional food

7.1. Curcuminoids

Curcumin (diferuloylmethane) is one of the major phytochemicals (70–80%, w/w) from the golden spice turmeric *Curcuma longa* Linn (family Zingiberaceae). The three main constituents of curcuminoids are curcumin, demethoxycurcumin, and *bis*-demethoxycurcumin, of which the important molecular structure for biological activity is diketone moiety (**Figure 5**).

Apparently, curcumin and its metabolites including di-, tetra-, and hexa-hydrocurcumin exhibit strong antioxidant, free-radical scavenging, anti-lipid peroxidative, antithrombotic, and anti-inflammatory activities. Many clinical investigations have addressed pharmacokinetics, safety (maximum dose 12 g/day over 3 months), and efficacy of this attractive nutraceutical against several human diseases including β-thalassemia. Many formulations of curcumin including nanoparticles, liposomal encapsulation, emulsions, capsules, tablets, and powder are available for a single and adjunctive treatment [106]. Curcumin is claimed to be a potential hexadentate iron chelator and found to remove NTBI in thalassemia serum and also suppress the ROS generation and lipid peroxidation in thalassemia RBC [83, 107–111]. Curcuminoids (particularly *bis*-demethoxycurcumin) and its metabolite (hexahydrobisdemethoxycurcumin) potentially enhanced the upregulation of γ-globin gene and synthesis of Hb F in human erythroid leukemia (K562) and primary erythroid precursor cells [112]. Curcumin is reported one of the triggers of eryptosis to allow defective RBC to escape hemolysis [8]. The oxidative stress condition in circulating RBC of TE patients is reduced after treatment with a curcumin cocktail, leading to improvement in their quality of life [83]. Curcumin markedly decreased iron deposition and lipid peroxidation product as MDA in the liver and spleen and the liver of iron-loaded rats [113].

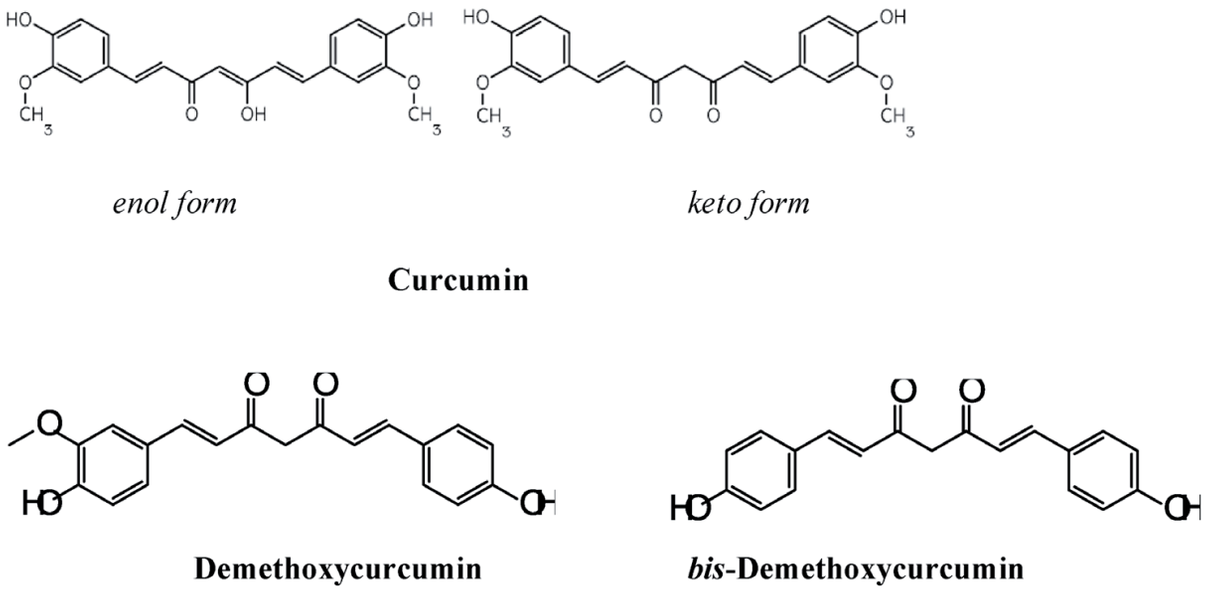

Figure 5. Structures of ingredients in curcuminoids.

7.2. Green tea

Tea (*Camellia sinensis* L., Theaceae family) is one of the most popular beverages in the world in which the products, depending on duration of fermentation, can be classified into green tea (GT), oolong tea, white tea, yellow tea, black tea, pu-erh tea, and Miang tea. GT (*C. sinensis* L. var japonica) is produced without any fermentation (oxidation), so the major persisting catechins are not destroyed by naturally occurring polyphenol oxidase (PPO) in fresh tea leaves. Oolong tea (*C. sinensis* var sinensis) is processed from tea leaves under semi-fermentation, in which β-glycosidic aroma precursors including 8-hydroxygeranyl β-D-primeveroside, *trans*- and *cis*-linalool 3,6-oxide 6-O-β-D-xylopyranosyl-β-D-glucopyranosides, and (2R,3S,4S,4aS,11bS)-3,4,11-trihydroxy-2-(hydroxymethyl)-8-(4-hydroxyphenyl)-3,4,4α,11β-tetrahydro-2H,10H-pyrano[2′,3′:4,5]furo[3,2-g]chromen-10-one are the main volatile constituents besides the catechin derivatives. Black tea (long fermentation) and Miang tea (*C. sinensis* L. kuntze var assamica) require very long fermentation times depending on the manufacturing process. Miang (a northern Thai word) is a chewing tea and commonly used for gum chewing in elderly people, relief of skin burn and inflammation, and as an antidiarrheal remedy.

In industry, GT is produced from steaming or roasting fresh tea leaves at high temperatures, consequentially drying and inactivating the PPO enzymes and leaving polyphenols known as flavonols or catechins at 30–40% by weight of dry tea leaves. It contains at least four major catechin derivatives including (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), and (−)-epicatechin (EC), of which the lipophilic permeable EGCG exhibits anti-oxidative and iron-chelating activities (**Figure 6**) [114]. Additionally, other phenolic acids including chlorogenic acid (CGA), caffeic acid (CA), and gallic acid (GA) and flavonols including kaempferol, myricetin, and quercetin are present in green tea [115]. Green tea extract (GTE) and EGCG, which show iron-chelating and antioxidant properties [116, 117] decrease labile iron (e.g., NTBI and LPI) level and consequently deplete lipid peroxidation as well as oxidative stress in both iron-loaded rats and thalassemia mice [118, 119]. The compounds were effective in the inhibition of RBC hemolysis, resulting in a prolonged RBC lifespan and decreased iron deposition and oxidative damage in the liver [119].

TI showed higher intestinal nonheme iron absorption than TM, while tea produced 41–90% inhibition of iron absorption in these patients, suggesting that tea consumption would be

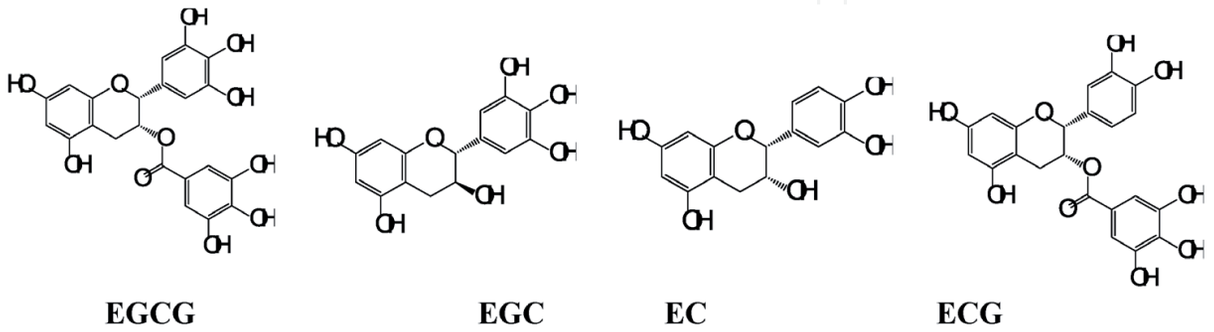

Figure 6. Structures of catechins in green tea.

recommended to thalassemia patients, particularly TI patients [120]. Logically, anti-oxidative GTE interferes duodenal absorption of dietary iron and iron-chelating properties in vitro and in vivo [116–119]. The preparation also showed inhibitory effect on catecholamine secretion from isolated rabbit adrenal glands, possibly by blocking L-type calcium channels in the adrenal medullary glands [121]. Therefore, GTE might be helpful to decrease iron deposition, reduce ROS levels, and ameliorate functions of targeted endocrine glands (e.g., pancreas and adrenal cortex) in β-thalassemia models. In controversy, a study reports development of thrombotic thrombocytopenic purpura in a person after consuming a weight-loss product containing green tea [122]. Most importantly, green tea showed antithrombosis ex vivo and inhibition of cyclooxygenase 1 activity [123, 124].

In our recent study, we have produced a functional GT-CUR concentrate (**Figure 7**) for investigating its effects in Thai adult TDT patents. We found that the drink did not affect white blood cell and platelet numbers, Hb, and Hct but increased RBC numbers following daily consumption for 2 months. The levels of blood urea, serum alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase activity tended to decrease but neither significantly nor dose dependently. In month 1 and 2 of the treatments, there were a decrease of serum MDA (−0.07 ± 2.95 and −0.87 ± 1.68 μM, respectively), NTBI (−1.20 ± 8.03 and −3.93 ± 3.83 μM, respectively), and LPI (1.91 ± 4.99 and −1.10 ± 2.94 μM, respectively) and increase of serum antioxidant activity (5.08 ± 8.86 and 0.28 ± 13.39 mg trolox equivalent/ml, respectively). These findings suggest GT-CUR drink would increase erythropoiesis, improve liver and kidney function, and diminish oxidative stress and iron overload in thalassemia patients [125]. Surprisingly, we demonstrate that treatment of GTE (1–10 μM EGCG equivalent) decreased cellular iron approximately 45% and ROS level in a concentration-dependent manner in iron-loaded pancreatic cell line (RINm5F) when compared with control cells. Secretory insulin level was nearly 2.5-fold times the highest safe concentration of the GTE [36]. The results imply that catechin-rich GT would indeed be an effective drink to remove iron, decrease ROS, and improve pancreatic cell function thereby increasing insulin production, leading to the amelioration of diabetic complications in thalassemia patients with iron overload.

Evidently, green tea is abundant with phytonutrient and enriched with active phytochemicals that exhibit many biological and pharmacological activities and it can be utilized for a functional

Figure 7. GT-CUR concentration: from field to nutraceutical product.

drink and health benefits. Up to now, many green tea products are being marketed worldwide for many purposes in different population ages. We are eager to use our multifunctional cocktail containing green tea extract, DFP, and vitamin E to examine if the product could diminish hypercoagulability and excessive platelet activity in thalassemia patients and thrombosis-related diseases, besides iron chelation.

7.3. Coffee

Coffee is also one of the most widely consumed beverages in the world because they contain many active ingredients that are a benefit for human health. Coffee (*Coffea arabica* L., *Coffea canephora* L. family Rubiaceae) is an original crop that will be further processed to roast coffee, coffee powder, coffee brew, coffee biscuit, and coffee candy for commercial purposes. Coffee is widely naturalized in many parts of the world including Africa, Latin America, the Pacific and Caribbean Ocean, Southeast Asia, and China. In Thailand, coffee is usually cultivated on the highlands at Doi Chang and Huay Nam Khun of Chiang Rai, Doi Saket District of Chiang Mai, and Kraburi District of Ranong (**Figure 8**).

Caffeine (1,3,7-trimethylxanthine) is a predominant ingredient persisting in tea and coffee, which is the most widely used pharmacologic substance showing prooxidant and antioxidant and hydroxyl radical scavenger [126–128]. Coffee contains many kinds of monosaccharide including sucrose, polysaccharides, D-arabinose, D-mannose, D-glucose, D-galactose, D-rhamnose, and D-xylose in nearly equal amounts. The amounts of caffeine and CGA are slightly higher in raw arabica coffee (0.9–1.2% and 1.6–2.4% w/w, respectively) than in raw robusta coffee (5.5–8.0% and 7.0–10.0% w/w, respectively) [129]. Interestingly, only arginine and cysteine are much more abundant in the green coffee (3.61% and 2.89% for arabica 2.28% and 3.87% for robusta) when compared with the roast coffee (0% and 0.76% for arabica 0% and 0.14% for robusta). Phenolic compounds including mono- and di-caffeoylquinic acids, CA, ferulic acid, *p*-coumaric acid, sinapic acid, 4-hydroxybenzoic acid, and CGA were detected in spent coffee by-product [130, 131] (**Figure 9**). Phenolic compounds, in particular CGA in coffee was able to chelate metal ion such as Zn [132]. In controversy for CVD incidence, one

Figure 8. Coffee crop in Thailand.

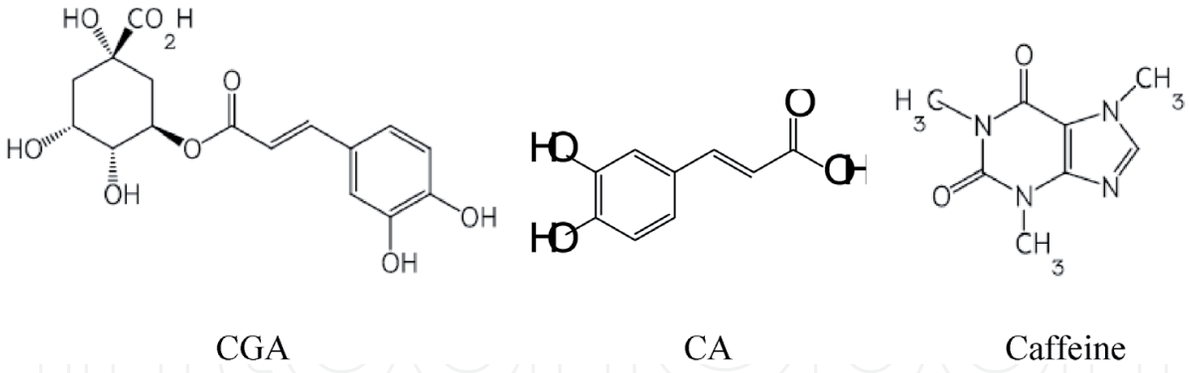

Figure 9. Some major constituents in coffee.

report supports the administration of caffeine augments endothelium-dependent vasodilation in young healthy volunteers through an increase in NO production [133]. Approximately one third of CGA and almost all of the CA are absorbed in the small intestine of humans, so the two antioxidants might have preventive effect of CVD [134]. The 10-kDa or less fractions but no other common components (e.g., CGA, CA, caffeine, quinic acid, trigonelline hydrochloride, and 5-(hydroxymethyl)-2-furfural) in hot-water extract of coffee had antithrombin and antiplatelet activity [135]. CGA protected oxidative damage and dose dependently increased the production of NO of human aortic endothelial cells [136].

Caffeine increases intracellular calcium-stimulating endothelial NOS to accelerate the production of NO which will be diffused to vascular smooth muscle cell to produce vasodilation [137]. Tocopherols are found in coffee bean oil [138]. Caffeine (300 mg, equivalent to two to three cups) is metabolized in the human body to theophylline (170 ng/ml plasma) 7 hours post-administration [139]. Tea and coffee dose dependently inhibited absorption of nonheme iron of either animal or plant food [140]. Dihydrocaffeic acid, a metabolite of CA detected in human plasma following coffee ingestion, was able to decrease ROS and increase NOS activity in human-derived EA.hy926 endothelial cells [141]. Ingestion of green coffee extract for 4 months led to the decrease in plasma level of homocysteine and improvement of human vessel reactivity [142]. Coffee ground residual has higher phenolic contents than roast coffee bean and shows inhibitory effects on the production of NO and pro-inflammatory cytokines in the macrophage [143]. Surprisingly, healthy volunteers who consumed coffee for 2 months (420 and 780 mg CGA equivalent/day) showed increase of plasma total antioxidant capacity [144]. A recent study has demonstrated coffee would counteract cerebral arterial constriction via endothelial NOS induction and smooth muscle dilation [145]. Two catechols, particularly CGA and CA which is abundant in coffee, could potentially scavenge free radicals and subsequently inhibit the production of pro-inflammatory cytokines as interleukin-8 in intestinal epithelial cells [146]. Consistent with our previous study, healthy adults consuming CGA-enriched coffee showed a significant increase of plasma antioxidant capacity when compared with the control group [144, 147]. Additionally, CGA-enriched green and roast coffee can protect oxidative damage of biomolecules in human consumers [148].

Nowadays, there are varieties of coffee products including green coffee powder, green coffee capsules, green coffee extracts, green coffee cleans detox, roast coffee, roast coffee, coffee brew, and herbal coffee that are commercially available for all-level consumers. In socioeconomics, the coffee beverage business is very popular and a growing industry in Thailand. We are

applying the wonderful properties of coffee for health benefits in thalassemia patients regarding anti-oxidation, metal chelation, and antithrombosis.

8. Fruits and vegetables

Epicarp extracts of bergamot (*Citrus bergamia* Risso) containing "citropten" and "bergapten" powerfully induced the expression and differentiation of γ-globin gene in human erythroid cells (K562) and consequently the production of Hb F, suggesting a potential therapy application in β-thalassemia and sickle cell anemia [149]. Fermented papaya preparation (FPP) increased the glutathione levels in blood cells and platelets and also decreased erythrocyte ROS level and membrane lipid peroxidation product levels such as MDA and phosphatidylserine in β-thalassemia patients [82, 150]. Mango (*Mangifera indica* L., family Anacardiaceae) is a tropical edible fruit cultivated globally and is annually produced from March to May. The seasonal fruit gives a high yield in Thailand and can be consumed in the forms of green and yellow fruits. It was found that aqueous extracts of the stem barks and peel from Vimang mango displayed potent antioxidant, free-radical scavenging and divalent metal ion-chelating properties due to the presence of a major polyphenol "mangiferin" [151]. Consistently, our group demonstrated that aqueous and ethanolic extracts of Thai mango (*M. indica* var Mahachanok and *M. indica* var Kaew) lowered plasma levels of glucose and triglyceride in streptozotocin-induced rats. Obviously, the extract showed analgesic, anti-gastric ulcerative, and chemical-induced hepatoprotective effects in rats. In addition, the extracts increased plasma antioxidant capacity in rats and humans [152]. The results suggest fresh and fermented mangoes would be a potential functional and therapeutic food against the deleterious action of ROS generated during iron overload (e.g., β-thalassemia, Friedreich's ataxia, hemochromatosis, and inflammation).

Rice (*Oryza sativa* L.) is the chief economic crop cultivated in every region of Thailand. One study demonstrated that consumption of wheatgrass juice and tablets decreased the requirement of RBC transfusions in Indian β-thalassemia patients by 25% or more [153, 154]. It was possible that pheophytin compound in the wheatgrass would increase hemoglobin synthesis. In controversy, another study showed that the juice therapy did not affect the production of hemoglobin [155]. Pancytopenia such as leukocytopenia and thrombocytopenia is observed in the chelation treatment of thalassemia; however, herbs like wheatgrass, papaya leaves, and garlic would be effective in treating single lineage cytopenias [156]. We found that ethanol extract of neem (*Azadirachta indica* var siamensis Valeton) leaves displayed free-radical scavenging and iron-binding activities in vitro, and the study of the extract will be extended to β-thalassemia mice with iron overload [157].

9. Conclusions

Regular iron chelation therapy with high dietary intake of antioxidants effectively lowers the harmfulness of iron overload-mediated oxidative tissue damage and organ dysfunctions in thalassemia patients. The supplementation with single nutrients, like antioxidants, is generally not effective in ameliorating such iron overload conditions or in slowing the progression

of the disease. It is recommended that these nutrients should be consumed as part of a healthy diet/functional fruits in daily meals. Nutritional and herbal strategies for modifying the pathological and clinical courses of thalassemia disease should consider the major active ingredients, nutraceuticals, biological activities, and hematological efficacy. Moreover, pre-implant/prenatal detections of thalassemia in the fetus using sensitive and specific molecular-biological and ultrasonic techniques could block new cases and problematic carriers of hemoglobinopathies. Understanding the genetics underlying the heritable subphenotypes of thalassemia would be prognostically useful and inform us further about personalized therapeutics as well as help the discovery and development of new pharmacogenomics. An effective medical regime, adjunctive supplementation of synthetic and natural antioxidants, and caregiver education could also be important factors to prevent or treat symptoms/complications in thalassemia. Selected protocols using single or combined chelators could be designed for personalized iron chelation therapy in TDT and NTDT patients, which would effectively and safely remove all the excess toxic iron (e.g., NTBI, LPI, and LIP) and prevent cardiac, liver, and other organ damage. Finally, a reliable approach based on genomics and proteomics may be effective to build a rational personalized medicine framework that can be applied in the preclinical, clinical, and therapeutic settings of hypercoagulability in thalassemia.

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Symbols and abbreviations

α	alpha
β	beta
γ	gamma
ADP	adenosine diphosphate
BKO	beta-knockout
CA	caffeic acid
CAT	catalase
CGA	chlorogenic acid
CS	Constant Spring
CVD	cardiovascular diseases

DFO	desferrioxamine
DFP	deferiprone
DFX	deferasirox
EC	(−)-epicatechin
ECG	(−)-epicatechin-3-gallate
EGC	(−)-epigallocatechin
EGCG	(−)-epigallocatechin-3-gallate
EPO	erythropoietin
FPP	fermented papaya preparation
G6PD	glucose-6-phosphate dehydrogenase
GA	gallic acid
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GST	glutathione-*S*-transferase
GT	green tea
GTE	green tea extract
Hb	hemoglobin
Hb A	adult hemoglobin
Hb E	hemoglobin E
Hb F	fetal hemoglobin
Hb S	hemoglobin sickle
Hct	hematocrit
HPFH	hereditary persistent fetal hemoglobin
HU	hydroxyurea
IRPs	iron-regulatory proteins
LIP	labile iron pools
LPI	labile plasma iron
MDA	malonyldialdehyde
MDS	myelodysplastic syndrome

MPs	microparticles
NAC	*N*-acetylcysteine
NO	nitric oxide
NOS	nitric oxide synthase
NTBI	non-transferrin-bound iron
NTDT	non-transfusion-dependent thalassemia
POD	peroxidase
PPO	polyphenol oxidase
PS	phosphatidylserine
RBC	red blood cells
RE	reticuloendothelial
ROS	reactive oxygen species
SAE	Southeast Asia
SCD	sickle cell disease
SOD	superoxide dismutase
TDT	transfusion-dependent β-thalassemia
thal	thalassemia
TI	β-thalassemia intermedia
TIBC	total iron-binding capacity
TM	β-thalassemia major
vWF	von Willebrand factor
XO	xanthine oxidase

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