GLUTATHIONE METABOLISM IN NORMAL SUBJECTS AND β-THALASSEEMIA/Hb E PATIENTS

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ABSTRACT

In Thailand, the β-thalassemia/Hb E disease is commonly found to result from the interaction between β^E-globin variation and β-thalassemia. The reduction of β-globin chain synthesis leads to an increase of unbound α-globin chain, unstable hemoglobin, hemolysis and releasing of free iron. This iron is the major source of oxidative stress. Free radicals or reactive oxygen species (ROS) generated by iron in Fenton's reaction can damage lipid membrane, protein and DNA. Human cells use enzymatic and non-enzymatic antioxidants to protect against oxidative stress. Glutathione (γ-glutamylcysteinylglycine; GSH) is the first line of protection against ROS. Reduced glutathione (GSH) is present mainly in the cells and can be converted to oxidized form (GSSG) during oxidative stress.

The oxidative stress and antioxidant status were studied in normal subjects (n=30) and β-thalassemia/Hb E patients (n=30). The protein carbonyl level was analyzed in plasma samples by spectrophotometry. We evaluated the total glutathione concentration (tGSH), GSH and GSSG by using high performance liquid chromatography (HPLC) with fluorescent detection. Furthermore, the catalytic activity of glutathione S-transferase (GST), glutathione peroxidase (GPX) and glutathione reductase (GR) were analyzed in erythrocytes of normal subjects and patients. The presence of oxidative stress was demonstrated by calculating redox ratio (GSH/GSSG).

Our findings showed statistically significant higher level of plasma protein carbonyl in splenectomized patients than non-splenectomized patients and normal subjects. The concentration of whole blood GSH was significantly decreased by 49% in patients. An increase GSSG was found in the patients resulting in reduction about 2.7-folds of redox ratio. Activities of GST, GPX, and GR were significantly increased in the β-thalassemia/Hb E patients (p <0.001).

The results suggest that the β-thalassemia/Hb E patients undergo oxidative stress resulting in the impairment of glutathione metabolism and antioxidative activities.

KEY WORDS : BETA-THALASSEMIA/HbE / OXIDATIVE STRESS / GLUTATHIONE METABOLISM / PROTEIN CARBONYL

150 pp.

กลูตาไธโอนเมตาบอลิสมในคนปกติ และผู้ป่วยบีต้าธาลัสซีเมีย/ฮีโมโกลบินอี
(GLUTATHIONE METABOLISM IN NORMAL SUBJECTS AND β-THALASSEMIA/Hb E PATIENTS.)

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บทคัดย่อ

บีต้าธาลัสซีเมีย/ฮีโกลบินอี เป็นโรคที่พบได้ในประเทศไทย เกิดจากมิวเตชั่นระหว่างมิวเตชั่นบนสายบีต้าโกลบิน กับธาลัสซีเมีย การผลิตโกลบินสายบีต้าไม่ได้นั้นทำให้โกลบินสายแอลฟามีมากเกินจนทำให้เกิดฮีโมโกลบินที่ไม่เสถียร และมีการปล่อยเหล็กออกเป็นอิสระ ซึ่งเป็นสาเหตุหลักของการเกิดของสภาวะ oxidative stress อนุมูลอิสระ หรือ อนุพันธ์ออกซิเจนที่ว่องไว (ROS) เกิดขึ้นจากปฏิกิริยา Fenton ทำให้เกิดความเสียหายต่อไลปิดเมมเบรน, โปรตีน และ ดีเอ็นเอ สารต้านอนุมูลอิสระที่เป็นเอนไซม์และไม่ใช่เอนไซม์ถูกใช้เพื่อป้องกันการเกิดสภาวะ oxidative stress กลูตาไธโอน (γ-glutamylcysteinylglycine; GSH) เป็นด่านแรกในการป้องกันอนุมูลอิสระ ในเซลล์พบรีดิวส์กลูตาไธโอนเป็นส่วนใหญ่ (GSH) และถูกเปลี่ยนเป็นออกซิไดซ์กลูตาไธโอน (GSSG)ในภาวะ oxidative stress

ในงานวิจัยครั้งนี้ ได้วิเคราะห์สภาวะ oxidative stress และสารต้านอนุมูลอิสระในคนปกติ และในผู้ป่วยบีต้าธาลัสซีเมีย/ฮีโมโกลบิน อี กลุ่มละ 30 คน โดยทำการวิเคราะห์ระดับโปรตีนคาร์โบนิลในพลาสมาโดยใช้เครื่องวัดค่าความทึบแสง วิเคราะห์ระดับกลูตาไธโอนทั้งหมด, GSH และ GSSG ในเลือดครบด้วย HPLC นอกจากนี้ได้ทำการวิเคราะห์แอคติวิตี้ของเอนไซม์ GST, GPX และ GR ในเม็ดเลือดแดง รวมทั้งอัตราส่วน GSH/GSSG (redox ratio) ซึ่งเป็นค่าบอกถึงสภาวะ oxidative stress

จากการทดลองพบว่าระดับโปรตีนคาร์บอนิลในพลาสมาของผู้ป่วยบีต้าธาลัสซีเมียที่ตัดม้ามมีระดับสูงขึ้นมากกว่าในคนไข้ที่ไม่ตัดม้ามและคนปกติอย่างมีนัยสำคัญ ระดับกลูตาไธโอนในเลือดครบของผู้ป่วยบีต้าธาลัสซีเมีย/ฮีโมโกลบิน อี ลดลงอย่างมีนัยสำคัญถึง 47% ระดับ GSSG ที่สะสมเพิ่มขึ้นในผู้ป่วยเป็นผลให้ระดับ redox ratio ลดลงประมาณ 2.7 เท่า ในขณะที่เอนไซม์ GST, GPX และ GR ในผู้ป่วยมีค่าแอคติวิตี้สูงขึ้นอย่างมีนัยสำคัญทางสถิติ

ในการศึกษานี้พบว่าผู้ป่วยบีต้าธาลัยซีเมีย/ฮีโมโกลบิน อี ที่อยู่ภายใต้สภาวะ oxidative stress มีการเปลี่ยนแปลงเมตาบอลิสมของกลูตาไธโอน และระดับสารต้านอนุมูลอิสระ

150 หน้า.

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LIST OF ABBREVIATIONS

α	alpha
β	beta
γ	gamma
AD	Alzheimer's disease
Arg	arhinine
CAT	catalase
CBC	complete blood count
CDNB	1-chloro-2,4-dinitrobenzene
CO_2	carbon dioxide
C=O	carbonyl group
Cu^{2+}	copper
k_d	dissociation activity
DNA	deoxyribonucleic acid
DTNB	5,5′-dithiobis-(2-nitrobezoic acid)
DNPH	2,4-Dinitrophenylhydrazine
DTT	dithiothreitol
EDTA	ethylenediamine tetrachloroacetic acid
$Fe2^{+}$	ferrous ion
$Fe3^{+}$	ferric ion
G6PD	glucose-6-phophate dehydrogenase
GCS	glutamylcysteine synthetase
GPX	glutathione peroxidase
GPX-GI	gastrointestinal GPX
GR	glutathione reductase
GS	glutathione synthetase
GSH	glutathione

LIST OF ABBREVIATIONS (continued)

GSSG	oxidized glutathione
GST	glutathione S-transferase
Hb	hemoglobin
HCl	hydrochloric acid
Hct	hematocrit
His	histidine
HIV	human immunodeficiency virus
$HO^{\bullet}$	hydroxyl radical
HOCl	hypochlorous acid
HPLC	High-performance liquid chromatography
HNE	4-hydroxy-2-nonenal
H_2O_2	hydrogen peroxide
LOOH	lipid hydroperoxide
Lys	lysine
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean cell volume
MDA	malondialdehyde
MPO	myeloperoxidase
NADPH	nicotinamide adeninedinucleotide phosphate
NEM	*N*-ethylmaleimide
NH_3	ammonia
NOS	nitrogen reactive species
NTBI	non-transferrin bound iron
NS	non-splenectomized
O_2	oxygen
$O_2^{\bullet-}$	superoxide radical
O_2^{2-}	peroxide ion

LIST OF ABBREVIATIONS (continued)

$^1O_2$	singlet oxygen
Plasma GPX	GPX-P
PHGPX	phospholipids hydroperoxide GPX
Pro	proline
RBC	red blood cells
$RO_2^{\bullet}$	peroxyl radical
$RO^{\bullet}$	alkoxyl radical
$RS^{\bullet}$	thinyl radical
ROS	reactive oxygen species
Ru5P	ribose-5-phosphate
SeOH	selenenic acid
SOD	superoxide dismutase
SP	splenectomized
tGSH	total glutathione
Thr	threonine
Trp	tryptophan
Tyr	tyrosine
uv	ultraviolet
WBC	white blood cells

CHAPTER I
INTRODUCTION

An inherited autosomal recessive disease in globin synthesis is called thalassemia. It occurs throughout the Mediterranean, the Middle East, the India subcontinent, Southeast Asia including Thailand (1). An imbalance of globin chain synthesis, defective of hemoglobin production, red cell precursor damage from excessive of globin subunit are most common condition in thalassemia. The defective of alpha (α)- or beta (β)-globin gene can be defined as α- or β-thalassemia. Ineffective alpha globin gene is often caused by deletion. The three deletion of α-globin gene leads to formation of β-globin chain tetramers or called hemoglobin H. The most severe of α-thalassemia is caused by complete α-globin genes deletion. In this condition, gamma (γ) globin gene becomes active in fetus and produces homotetramer of 4 gamma (γ) chains (Hb Bart's), which are unable to transport oxygen. The genetic deletion is a most commonly found in α-thalassemia, whereas point mutation and nucleotide substitution cause β-thalassemia. β-thalassemia which is prevalent in the Mediterranean countries, North Africa, The Middle East, and Southeast Asia (2, 3).

The β-thalassemia is divided into β-thalassemia minor (heterozygote gene), β-thalassemia intermediate, and β-thalassemia major (homozygote gene). The mild or no anemia can be observed in β-thalassemia minor. The β-thalassemia major has severe symptoms with no β globin chain production. The β-thalassemia is caused by 200 genetic different mutations. The hemoglobin E (point mutation encoding glutamine substitute of lysine at position 26 of β chains $\alpha_2\beta_2,26^{Glu \rightarrow Lys}$) gene can interact with β-thalassemia alleles and cause clinical severity ranging from β-thalassemia intermediate to β-thalassemia major (2-4). The interaction between hemoglobin mutation and β-thalassemia lead to β-thalassemia/Hb E, which is the most commonly found in Southeast Asia. The frequency of hemoglobin E is as high as 13% of total population and 50% in northeastern population in Thailand (5).

An excess of unpaired α-globin chains in β-thalassemia leads to red blood cell damage. The unstable hemoglobin, excess of α globin subunits, and high levels of cytosolic, membrane bound iron and under continuous blood transfusion treatment lead to an iron overload which are major source of free radical overproduction by thalassemia erythrocytes. The development of this pathology leads to chronic anemia and increase oxidative stress.

The iron exists as the heme and nonheme complexes of biomolecules such as hemoglobin, myoglobin, cytochromes, transferrin and ferritin. As iron loading process, the main transport protein of iron is serum transferrin which binds and detoxifies iron whereas plasma non-transferrin bound iron (NTBI) promotes the generation of free hydroxyl radicals. The role of increased iron absorption is demonstrated by the severe iron overload and an increase of catabolic iron from reticuloendothelial system which overwhelms the iron-carrying capacity of transferrin resulting in the emergence of toxic NTBI. The formation of hydroxyl or hydroxyl-like radicals in the reaction of ferrous iron (Fe^{2+}) and hydrogen peroxide by Fenton reaction is usually considered as a main mechanism of free radical damage. Iron toxicity is involved in various human pathologies such as acute iron poisoning, congenital transferrinemia, as well as secondary iron overload in β-thalassemia. Organic reactive species, such as peroxyl ($RO_2^{\bullet}$), alkoxyl ($RO^{\bullet}$) and thinyl ($RS^{\bullet}$) radicals are generated by iron (Fe^{2+}). The labile iron, plasma iron and directly chelatable iron serve as early indicators of iron overload β-thalassemia/Hb E patients (7). Serum ferritin and nontransferrin bound iron are detected in all thalassemia patients (8). Although, iron is important in cell metabolism and cell growth but the accumulation of iron must be eliminated quickly because excessive iron damages the liver, endocrine organs, and heart.

Free radicals are defined as molecular species which contain at least one unpaired electron. Oxygen molecule is essential for human life but it can be harmful by serving as a source of reactive oxygen spices (ROS). It becomes a free radical when a single electron adds to π antibonding orbital, superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) will be produced by this reaction. Reactive oxygen species is a group of oxygen radicals such as superoxide, hydroxyl ($OH^{\bullet}$), peroxyl ($RO_2^{\bullet}$)

and non radical, such as H_2O_2, hypochlorous acid (HOCl) and ozone (9). The oxidation-reaction of hemoglobin involves in Fenton reaction when the hydroxyl radicals and superoxide anion radicals are present. The formation of highly reactive hydroxyl is observed in β-thalassemia patients who showed four times higher than normal subjects (4). Furthermore, the chain-reaction of free radicals can attack other biomolecules including DNA, lipid, and protein.

Oxidative stress can cause double-strand break lesion in DNA. A sensitive micronucleus assay was used to measure the frequency of chromosomal breaks in patients with α- and β-thalassemia indicating that higher levels of micronuclei frequencies are present in thalassemic RBC (10). Polyunsaturated fatty acids (PUFA) and phospholipids in membrane are susceptible to free radicals which lead to lipid peroxidation reaction and cause transmembrane protein such as receptor or enzyme damage. Malondialdehyde (MDA) is a product of lipid peroxidation and used as a marker of oxidative stress. Furthermore, ROS can lead to oxidation of protein backbone, amino acid side chains, and protein fragmentation (11).

The ROS species are also generated in thalassemic red blood cells because the deposition of excess unmatched globin chains contains free iron, nonheme iron, and hemichromes. These compounds can generate ROS by several mechanisms including Fenton reaction (12). There are many evidences of oxidative stress which can be observed in β-thalassemia patients. A commonly biomarker of oxidative stress is MDA that found in high level in thalassemia patients (8, 13-16). The deficiency of antioxidant in β-thalassemia was due to its increased consumption in oxidative stress condition (17). However, the humans develop the protective process to against free radicals and ROS. The antioxidant systems can be divided to enzymatic and non-enzymatic antioxidants. Enzymatic antioxidant can be produced in the cell such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX). Non-enzymatic antioxidants can be obtained from food supply; fruits or vegetables such as vitamin E, vitamin C, and β-carotene.

One of the most effective antioxidant defense systems in human is glutathione which is the major of intracellular non-protein antioxidant that is produced in all organs, especially in liver. Glutathione plays a crucial role in detoxification of free radicals. The normal range of glutathione is from 1 to 10 mM in mammalian cells,

spleen, kidney, erythrocytes and being most concentrate in liver (up to 10 mM). Total glutathione in the cell is found in free form or protein bound form. Free form of glutathione is in a reduced form (GSH) that can be converted to oxidized form (GSSG) during exposed to oxidative stress. The redox status shows the ratio between reduced and oxidized form (GSH/GSSG). The low level of GSH and high in GSH/GSSG ratio has been found in blood of patients such as breast cancer, HIV infection, and lung cancer (18).

The functions of GSH are served as the first line of antioxidant pathway against ROS by scavenging free radicals. The second line of defense relates to enzyme activities which use GSH as substrate. These enzymes are glutathione peroxidase (GPX), glutathione reductase (GR) and glutathione S-transferase (GST). Glutathione has been used as a biomarker to study in various conditions such as a deficiency of GSH in hemolytic anemia, degeneration of spinocerebellar neuropathy, myopathy, and HIV infections (18). Antioxidant function of GSH also depends on its role as a component of the enzymatic pathway, consisting of glutathione peroxidase (GPX), which is a selenoenzyme, and its catalytic function depends on the presence of the mineral (selenium) in the enzyme at the active site (19). It has an ability to catalyze the conversion of hydrogen peroxide which is produced by SOD. The glutathione S-transferase (GST) family comprises a long list of cytosolic, mitochondrial, and microsomal proteins which have ability to detoxify many substrates including xenobiotic, endobiotic compounds, carcinogen, therapeutic drugs, and by-products of oxidative stress. Human GST are a functionally diverse the soluble enzymes of detoxification that use GSH in conjugation and reduction reaction (20). Glutathione reductase (GR) is a ubiquitous enzyme which catalyzes the reduction of GSSG to GSH. This enzyme is essential for glutathione redox cycle that maintains cellular GSH level. This homodimeric enzyme is a member of the family of flavoprotein disulfide oxidoreductase. The level of enzyme seems to be increased when exposed to oxidative stress.

Another marker that is the most commonly used for protein oxidation is protein carbonyl (21). Iron (Fe^{2+}) or copper (Cu^{2+}) can bind to protein on cation binding location and can further be attacked by H_2O_2 or O_2 and transformed side chains of amine groups (i.e., lysine, arginine, praline, and histidine) into carbonyls.

The protein carbonyl derivatives can also be generated through oxidative cleavage of protein by α-amidation pathways or oxidation of glutamyl side chains (22). Polypeptide backbone is oxidized by attacking of $OH^{\bullet}$ at amino acid residue to form an alkylperoxide and alkoxyl radical.

There are many studies involving the enzymatic and non-enzymatic antioxidants in thalassemia patients such as MDA, vitamin C, E, NTBI, iron level, and antioxidant enzymes e.g. CAT, SOD and GPX (9-12). The redox status of glutathione and protein carbonyl have been studied in many diseases such as tumor cell (23), aging (24), nonalcoholic steatohepatitis (25), Parkinson's disease (26), HIV infection (27), Down syndrome (28) except β-thalassemia/Hb E. Therefore, it is of interest to study the glutathione metabolism including GSH/GSSG ratio and glutathione-dependent enzyme and plasma protein carbonyl content in β-thalassemia/Hb E patients compared with healthy normal subjects.

Objectives

1. To determine glutathione metabolism and protein oxidation in β-thalassemia/Hb E patients and normal subjects.
2. To compare redox status from GSH/GSSG ratio by using HPLC and fluorometric techniques.

CHAPTER II

LITERATURE REVIEW

1. Human blood

Blood is a complex mixture containing plasma, white blood cell, red blood cell and platelet. Red blood cells or erythrocytes have limited metabolic function because its lack of internal organelles during cell differentiation. The erythrocyte can transport oxygen to the tissues. The white blood cells or leukocytes are nucleated cells that can act against infection. The platelets or thrombocytes contain cytoplasmic organelles, no nucleus and involving in blood clotting. Human's blood has life-span about 120 days, normal red cell count in adult men were 12.1-16.6 g/dl and whereas in adult women, there are about 12.1-15 g/dl (29).

2. Hemoglobin

Hemoglobin is a protein found in red blood cell. It transports oxygen from lung to rest of the body such as muscle (Figure 1). It consists of two pairs of α- and β-globin protein and forming as heterotetramer, each peptide chain subunit contains about 140-150 residues, which is formed α-helical pattern and bound to heme group. Heme is a large flat-iron containing structure. The heterocyclic ring system of the heme is a porphyrin derivative, which contains four pyrole rings link to methane bridge. The iron atom which is the site of oxygen binding, binds to four nitrogen center of the ring and globular protein via the imidazole ring of a histidine residue below the porphyrin ring (30-32). Normal human adult hemoglobin consists of hemoglobin A (HbA; $\alpha_2\beta_2$) 97%, hemoglobin A_2 (HbA_2; $\alpha_2\delta_2$) 2.5%, and less than 1% of hemoglobin F (HbF; $\alpha_2\gamma_2$) (1).

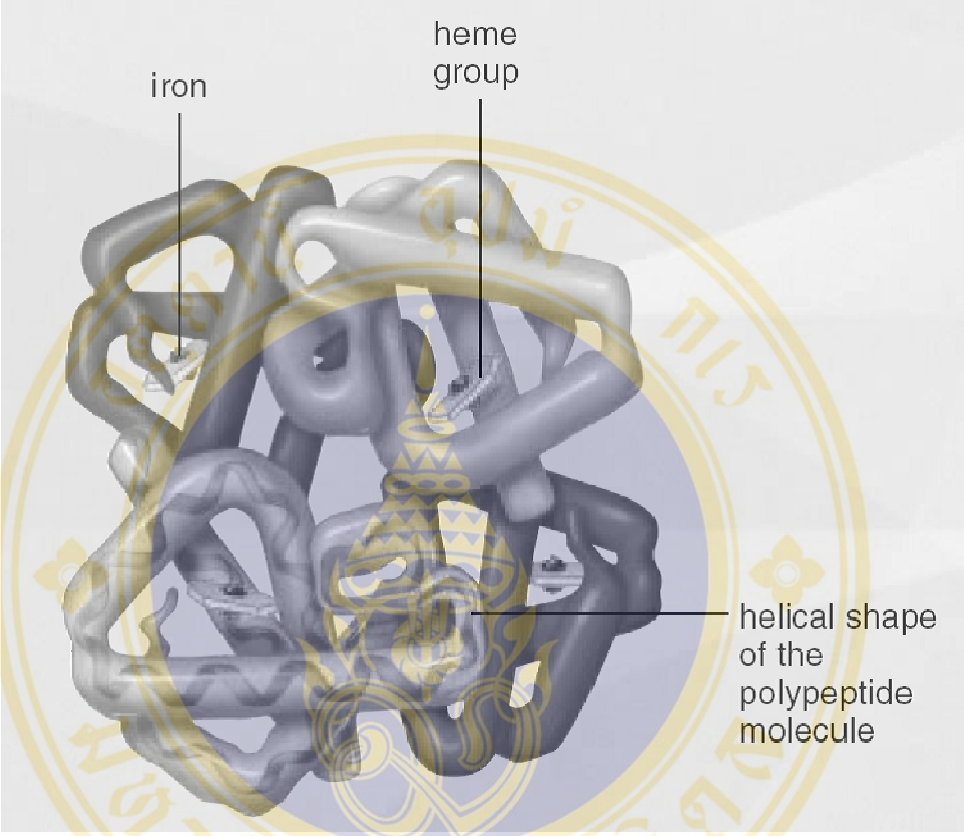

Figure 1 Hemoglobin structure (32)

3. Thalassemia

Thalassemia mainly consists of α- and β-thalassemia, that depend on which pair of globin chains is synthesized insufficiently. The β-thalassemia is caused by a decrease of β-globin chain production, which consequence from more than 200 different mutations; promoter mutation, nucleotide substitution and rarely deletion (33).

The mutation on β-globin genes results in either the absence of β-globin chain synthesis (β^0-thalassemia) or a reduction of synthesis (β^+-thalassemia). The severity of this disease can be used to classify a clinically severity as β-thalassemia major, intermediate and minor. The homozygous or heterozygous compound of β-thalassemia is call thalassemia major, which has a severe anemia and need blood transfusion. The β-thalassemia intermediate is more severe than minor but milder than major thalassemia. The β-thalassemia intermediate patients are able to maintain a hemoglobin level of approximately 6 g/dl without blood transfusion. The heterozygous state of β-thalassemia is asymptomatic; β-thalassemia miner has mild or no anemia. There is only one β-globin gene carrying a thalassemia mutation, which has no clinically sign of symptoms and does not need blood transfusion (1, 34).

Hemoglobin E (Hb E) is variant hemoglobin with a mutation in the β globin gene, most commonly found in Southeast Asia. The mutation is base substitution of glutamic acid for lysine at position 26 of the β-globin chain ($\alpha_2\beta_2^{26\,(Glu\rightarrow Lys)}$) (35). Hb E can be found in homozygous or heterozygouse state. The β-chain of hemoglobin E is synthesized at a reduced rate compared with normal adult hemoglobin (1). The frequency of Hb E is 13% in Thailand (6). The interaction of Hb E and thalassemia produces variable phenotypes ranging from mild to a transfusion-dependent thalassemia. The β-thalassemia/Hb E is commonly found in Southeast Asia including Thailand (6, 36-38). The hemoglobin level of β-thalassemia/Hb E diseases is approximately 7 g/dL (6).

The anemia in thalassemia is mainly caused by ineffective erythropoiesis (3). Unstable hemoglobin, excess of α-globin chains are incapable to form hemoglobin and it precipitates in the red cell precursors leading to their damage, in bone marrow and peripheral blood, a process call ineffective erythropoiesis (Figure 2).

The precipitated α-chains generate inclusion bodies and hemichromes. The hypercoagulable state in thalassemia is caused by amplifying thrombin generation and initiate platelet activation. Furthermore, the degradation of ineffective erythrocyte release iron, which effect to lipid membrane and increase membrane-bound hemichromes. The phospholipid membrane of thalassemic red cell increases adherence the phophatidylserine to attach endothelial cell and it enhances thrombin generation to activate platelet, granurocyte and monocytes (39). Spectrin is a protein in red cell membrane skeletal. The excess of α-globin in thalassemia patients interact directly to spectrin. Dissociation activity (k_d) of spectrin to hemoglobin in patient was lower than normal (40, 41). A modification on membrane band 3 protein or spectrin by oxidation is lead to membrane damage (Figure 3).

The iron overload and high level of ferritin was observed in thalassemia patients (42). The free radicals or reactive oxygen species (ROS) were generated by several mechanisms such as Fenton's reaction. Thalassemic red cells from β-thalassemia intermedia patients without blood transfusion can enhance the generation of $HO^{\bullet}$ greater than normal red cells (7, 15). The oxidation of lipid was measured as plasma malonyldialdehyde (MDA) level which was found to be higher in β-thalassemia patients than normal (43-45). Role of oxidant injury caused by ROS has effect on antioxidant enzymatic levels in thalassemic patients. Those antioxidant enzymes include superoxide dismutase (SOD), glutathione peroxidase (GPX), and glutathione reductase (GR). The antioxidant enzymes were measured in β-thalassemia patients (14, 45) and higher than normal. Oxidative stress in β-thalassemia patients may cause by membrane bound free iron, hemichrome and non-heme iron or methemoglobin, which found high level in red cell of splenectomized β-thalassemia intermediate patients (46). The oxidant injury caused by the presence of reactive oxygen species (ROS) in thalassemia diseases is one of the normal pathophysiology of this disease. Moreover, accumulation of iron in thalassemia patients is a primary cause of death. The deposition of iron occurs in heart, liver, endocrine gland and eventually is a cause of mortality in β-thalassemia (47).

4. Free radical and oxygen toxicity

Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in molecular orbital. They are unstable and very reactive (48). They can be represented as a molecular that contain radical, write as dot to the right preceding any charge in their chemical formula as well as when using abbreviations. This placement of the dot will not interfere with indication of mass number, atomic number, or composition (49).

Oxygen (O_2) is essential to human life and toxic. It is a biradical molecule that contains two unpaired electrons in separate orbital (Figure 4). Chemical characteristic of oxygen is due to the two unpaired electrons in the external π^* orbital with the same spin. This is the most stable or ground state of oxygen. However, when oxygen accepts single electrons in 4 step electron reduction, it is transformed into highly reactive oxygen radicals (Figure 5) that damage lipid, protein and DNA. When oxygen receives one electron, it forms superoxide radical ($O_2^{\bullet}$). Superoxide radicals arise through metabolic process or radiation exposure is considered the "primary" ROS and it can be interacted with other molecule to generate "secondary" ROS. The addition of 2 electrons to peroxide ion (O_2^{2-}) can generate hydrogen peroxide (H_2O_2). Hydroxyl radicals ($HO^{\bullet}$) can also be produced by hydrogen peroxide fission, Fenton's reaction (34). In animal cell, there are many pathways to generate ROS or nitrogen reactive species (NOS) as shown in Figure 6. Respiratory burst, lipid oxidation, auto-oxidation of hemoglobin or protein oxidation are source of ROS/NOS formation *in vivo* (50).

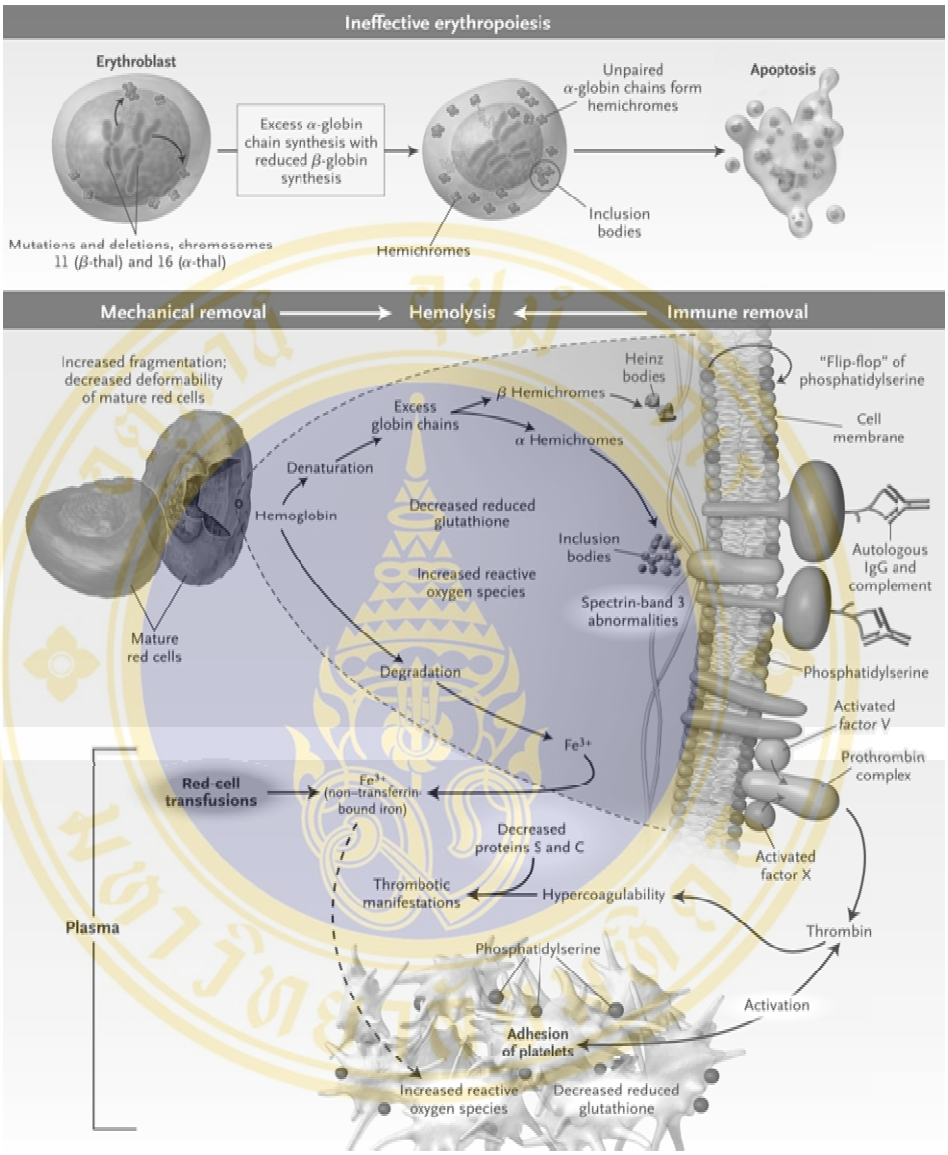

Figure 2 Ineffective erythropoiesis and hemolysis pathway in thalassemia (3)

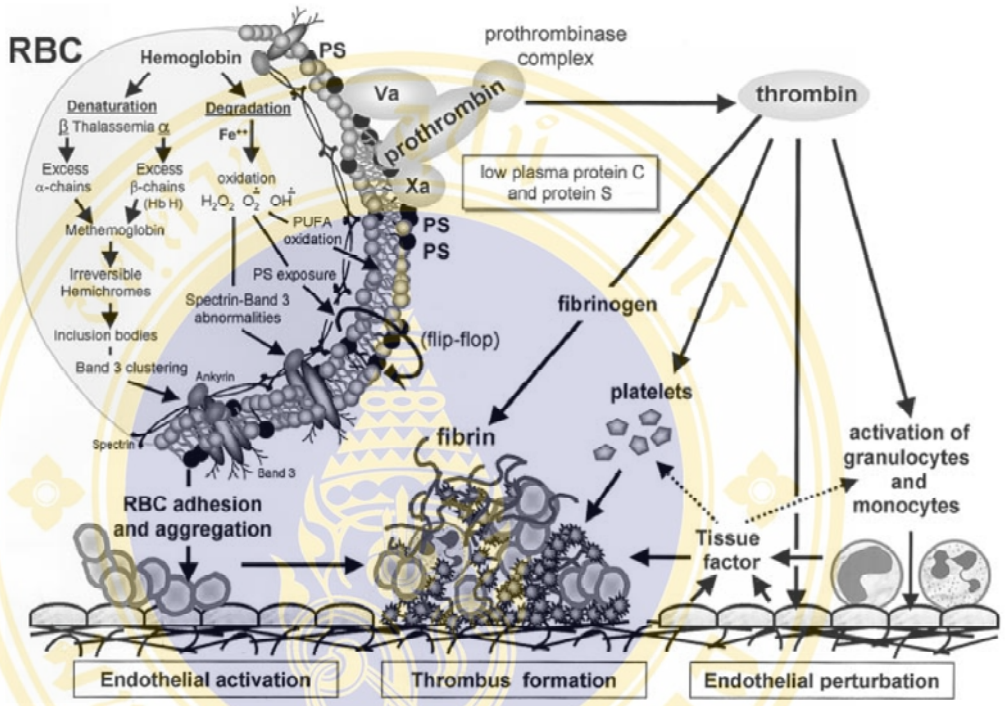

Figure 3 Hypercoagulable state of thalassemic red blood cell (39)

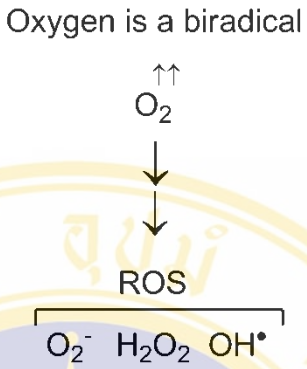

Figure 4 Biradical of oxygen (30). It has two antibonding electrons with parallel spins. It has a tendency to form toxic reactive oxygen species (ROS), such as $O_2^{\bullet -}$, H_2O_2, and $HO^{\bullet}$

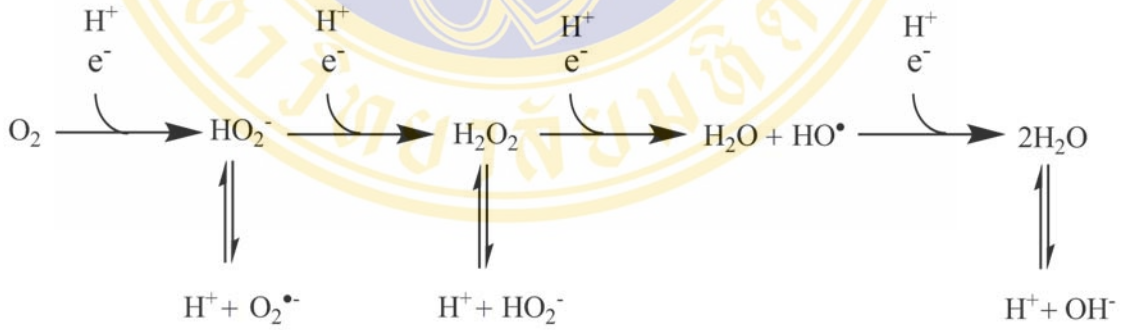

Figure 5 Reduction of oxygen by four-step one electron (30)

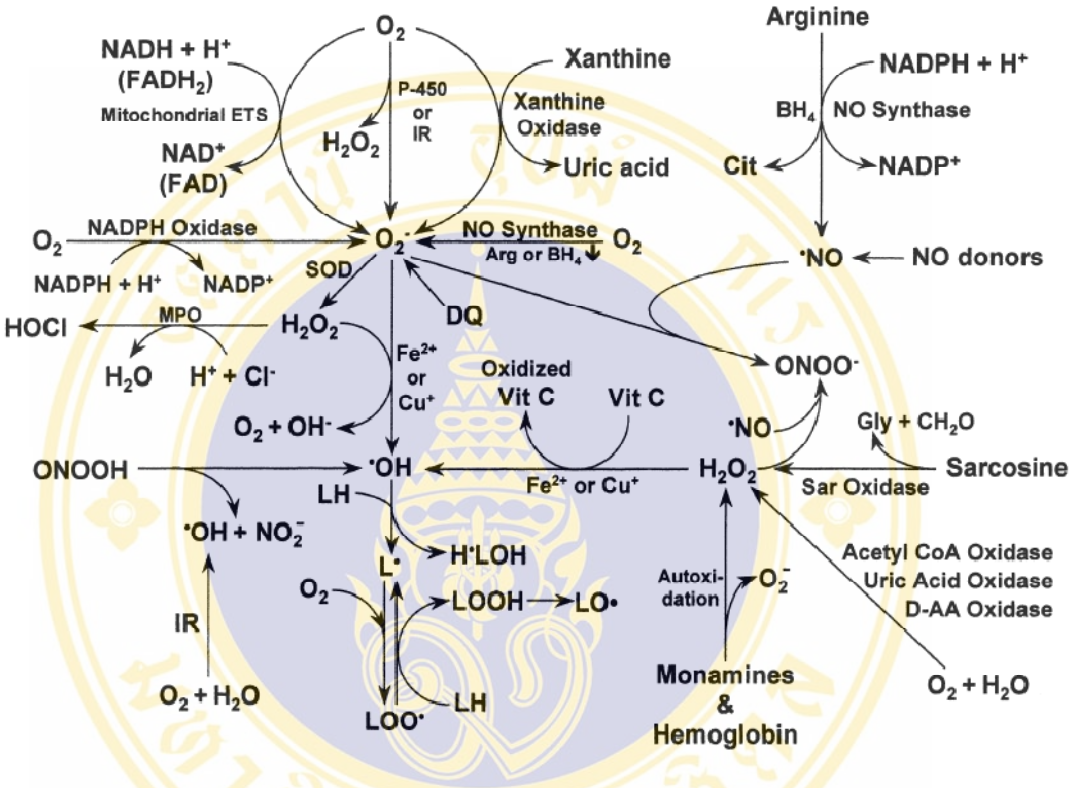

Figure 6 Production of oxygen and nitrogen free radicals and other reactive species in mammalian cells (51)

Table 1 Example of reactive oxygen species (34)

Reactive species	Properties
$O_2^{\bullet -}$ (superoxide anion)	Produced by the electron transport chain and at other sites. Cannot diffuse far from the site of origin. Generates other ROS
H_2O_2 (hydrogen peroxide)	Not a free radical, but can generate free radicals by reaction with a transition metal (e.g., Fe^{2+}). Can diffuse into and through cell membranes.
$HO^{\bullet}$ (hydroxyl radical)	The most reactive species in attacking biologic molecules. Produced from H2O2 in the Fenton reaction in the presence of Fe^{2+} or Cu^{+}
RO•, R•, R-S• (organic radicals)	Organic free radicals (R denotes remainder of the compound.) Produced from ROH, RH (e.g., at the carbon of a double bond in a fatty acid) or RSH by $HO^{\bullet}$ attack.
ROO• (peroxyl radical)	An organic peroxyl radical, such as occurs during lipid degradation
HOCl (hypochlorous acid)	Produced in neutrophils during the respiratory burst to destroy invading organisms. Toxicity is through halogenations and oxidation reactions. Attacking species is OCl^{-}
$^1O_2$ (singlet oxygen)	Oxygen with antiparallel spins. Produced at high oxygen tensions from absorption of uv light. Decays so fast that it is probably not a significant in vivo source of toxicity.

4.1 Superoxide ($O_2^{\bullet -}$)

Superoxide or superoxide anion ($O_2^{\bullet -}$) produces mostly within the complex I in mitochondria (Figure 7) (52-54). Superoxide anion is generated in respiratory chain at the inter membrane space because respiratory complex leak of electron to oxygen molecule (Figure 8) (55). It arises through metabolic processes or oxygen activation and further interacts with other molecules to generate other reactive species (Figure 6). Superoxide anion is a precursor of H_2O_2 which catalyze by superoxide dismutase (SOD).

$$2O_2^{\bullet -} + 2H^+ \xrightarrow{superoxide\ dismutase} O_2 + H_2O_2$$

Superoxide is produced in phagocytocytic respiratory burst (34) by NADPH oxidase (56), xanthine oxidase in the liver, small intestine and capillary endothelium can transfer electrons to molecular oxygen to form $O_2^{\bullet -}$. During normal cell metabolism, it was generated by pharmacological agent and rapidly react with NO to form peroxynitrite which is cytotoxic to the cell (57).

$$H_2O_2 + \text{xanthine} + 2O_2 \longrightarrow \text{uric acid} + 2O_2^{\bullet -} + 2H^+$$

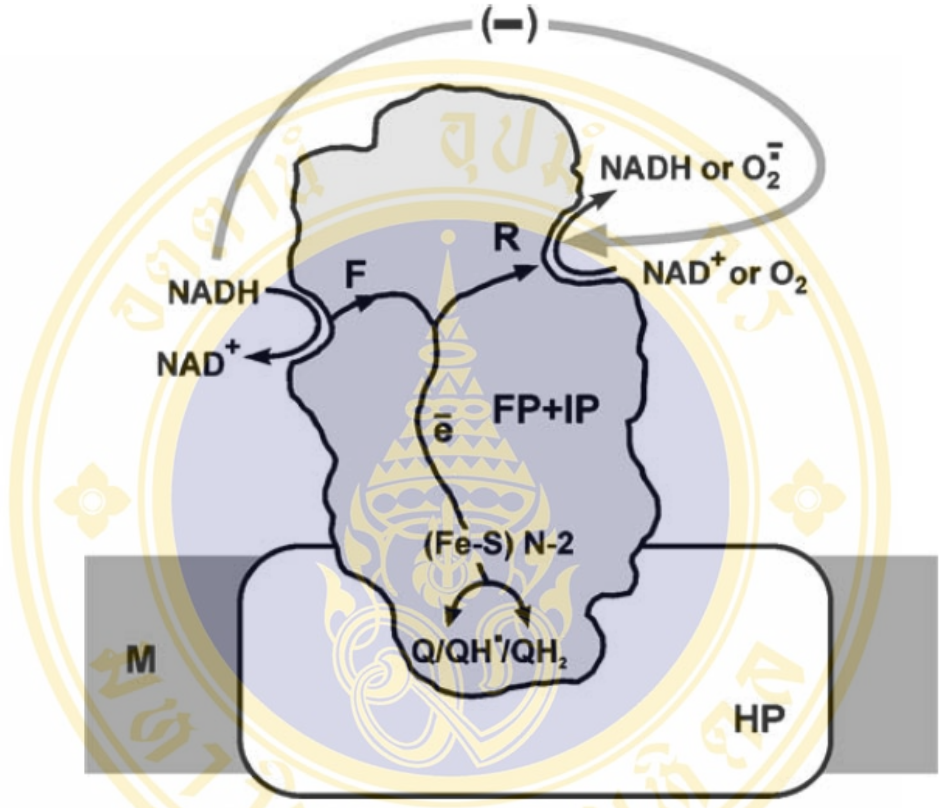

Figure 7 A model for electron transfer pathway in NADH oxidation (53)

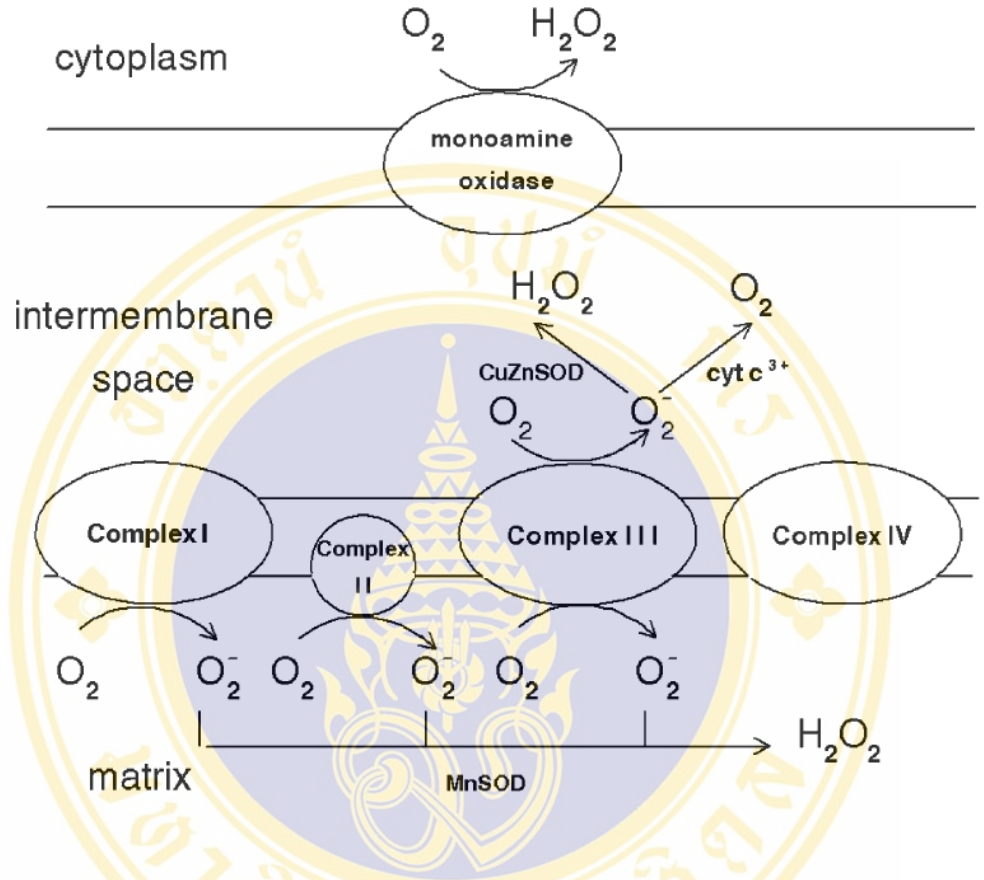

Figure 8 Sites of superoxide formation in the respiratory chain (55)

4.2 Hydrogen peroxide (H_2O_2)

Hydrogen peroxide is poorly reactive but it acts as intermediate to generate other reactive oxygen species and lipid peroxidation in biological system. It is a mild oxidizing or reducing agent. H_2O_2 was generated in biological system from superoxide anion. Enzyme xanthine oxidase use $O_2^{\bullet -}$ and reduces 80% of the O_2 as a source of hydrogen peroxide generation (58). Hydrogen peroxide is lipid soluble, it can diffuse through membranes and generate hydroxyl radical ($HO^{\bullet}$) at localized Fe^{2+} or Cu^{+} containing site, and can also be generated by ultraviolet light (59). Hydrogen peroxide is also the precursor of hypochlorous (HOCl), a powerful oxidizing agent that produced by phagocyte cells (34).

$$H^+ + Cl^- + H_2O_2 \xrightarrow{myeloperoxidase} HOCl + H_2O_2$$

The hypochlorous acid is formed from H_2O_2 and chloride, has been implicated in lipid peroxidation by phagocytes (60). Hydrogen peroxide can be found in human plasma very low because of the presence of catalase and it also reacts with heme protein, ascorbate and protein-SH group. *In vivo*, H_2O_2 can be generated and diffused through erythrocyte, white cells, endothelial cells and platelet for metabolism. Although, H_2O_2 level in human is very variable, but in human, H_2O_2 rapidly remove (59).

4.3 Hypochlorous acid (HOCl)

The phagocyte cell has ability to kill a wild range of pathogen by using myeloperoxidase-H_2O_2-Cl system (61). However, HOCl can distribute to tissue injury during inflammation. The oxidation of low-density lipoprotein (LDL) by HOCl produces a modified HOCl-LDL, which is induced platelet aggregation and α-granule secretion (62). Hyperchlorous acid can oxidize thiol, thioether of methionine, ascorbate, and tryptophan. The loss of thiol molecule by HOCl leads to decrease GSH in the cell (63).

4.4 Hydroxyl radical ($HO^{\bullet}$)

Hydroxyl radical is a highly reactive molecule, its can interact with various kind of biological molecule. It can be generated by chain reaction from ROS, Fenton's reaction, and Haber-Weiss reaction. As shown in Figure 6, ROS, hydroxyl radicals can also generated from hemolytic fission of hydrogen peroxide by radiation:

$$H_2O_2 \xrightarrow{radiation} 2HO^{\bullet}$$

The reaction between transition metal ions (ferrous iron) and hydrogen peroxide gives rise to hydroxyl radical via Fenton's reaction (64):

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

The reduction of $O_2^{\bullet-}$ to oxygen catalyze by Fe^{3+}. From the couple of Fenton's reaction and catalytic of $O_2^{\bullet-}$, the net reaction or call Haber-Weiss reaction can generate hydroxyl radical. In the presence of superoxide, hydrogen peroxide and transition metal ion are able to generate hydroxyl radical (65).

$$Fe^{3+} + O_2^{\bullet-} \longrightarrow Fe^{2+} + O_2$$

The net reaction:

$$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + HO^{\bullet} + OH^-$$

Hydroxyl radical has ability to react with a wide range of cellular constituents, for example, it damages DNA by interacting with amino acid base such as thymine and guanine which can be detected by using HPLC technique (66).

4.5 Singlet oxygen ($^1O_2$)

Oxygen in excited state is singlet oxygen ($^1O_2$). Singlet oxygen is generated by absorption of uv light (UVA, 320-400 nm) (67) or in the biological system of the cells (68); such as during oxidation of NADPH in liver microsome (69), and human eosinophil (70). It can generate highly in the reaction of ozone (O_3) (71). It has high electrophilicity, reactivity and long life time in water (72). It reacts rapidly with amino acid, unsaturated lipid, and can directly react with molecule in photo

sensitizer area. Singlet oxygen can also cause damage human lens in vitro (73). $^1O_2$ can damage protein by physical quenching and chemical reaction on protein side chain; tryptophan (Trp), tyrosine (Tyr), and histidine (His) (74). Furthermore, singlet oxygen damage DNA by interacting with nucleosides, which causes DNA lesion (75). There are many effects of singlet oxygen mediated oxidation protein. It can inactivate enzyme leading to protein peroxide formation, side chain or back-bone fragment and cross-link and aggregation of protein attacking of $^1O_2$ (76).

5. Source of free radicals

Aerobic respiration organism consume oxygen molecule to produce energy. During electron transport system, electron can leak out from mitochondria as a major source of ROS. Complexes I and III of the mitochondrial chain are the most important sites of ROS formation. Free radicals or ROS such as superoxide anion ($O_2^{\bullet -}$) and hydroxyl radical ($HO^{\bullet}$) are predominately in the cell. Superoxide is produced from both complex I and III of electron transport chain and rapidly transport cross the inner mitochondrial membrane (50). Furthermore, superoxide also has multiple pathways in production such as NADPH oxidation and action of xanthine oxidase. The highly reactive species, hydroxyl radical is attached to closely its site of formation. It produces during breakdown of hydrogen peroxide by transition metal ion (Fenton's reaction). Hydrogen peroxide is not reactive species but it also generates other radical molecule. In four-step electron, hydroxyl radical can produce by H_2O_2 (48). The oxidation of superoxide by enzyme SOD can produce hydrogen peroxide. Moreover, the uv light also generate singlet oxygen by attacking to oxygen molecule. The protective against the pathogen by phagocyte leads to produce a radical molecule by enzyme NADPH oxidase (77). In addition, the chemical reagents, environmental pollutants, xenobiotic agents, and some human diseases can generate reactive radicals which can damage the cells.

6. Oxidative stress and human diseases

The term of oxidative stress is an imbalance or shifts in the pro-oxidant/antioxidant status of a system. Cellular injury and pathologic change occur when a favoring oxidant over antioxidants within a living organism. Oxidative stress leads to damage the biological molecule. Various kinds of biological molecules are attacked from reactive species. The potentially effect of oxidative stress is to damage lipids, protein, carbohydrate, and DNA. Finally, the effect of oxidative stress ultimately leads to cell death (78). Oxygen radicals and other activated oxygen-species are generated by aerobic metabolism or toxicant expose. The radical species are the important intermediates in both normal as well as in a disease. ROS is a reactive and forming of toxic species such as peroxides, aldehydes, ketones.

Oxidative stress is an excess of free radicals produced in the cell. In human body, oxidative stress can generate through many mechanisms such as mitochondrial leakage, respiratory burst, pollutants, uv light, environmental factor, and carcinogens. An increase of oxidative stress in cell can be interrupted by antioxidant. However, the severe oxidative stress can lead to cell injury or cell death. Normally, humans tend to control the oxidative stress by using enzymatic and non-enzymatic antioxidants system. The balance between the rate of production of oxidants and the rate of clearance by several enzymes or antioxidant compounds has been used to monitor the redox homeostasis in the cells (48, 50, 79). If the rate of ROS production and destruction is balance, cell is in stable state (Figure 9). Oxidative stress acts as a central role in many diseases. There are many diseases that have been shown to be involved the oxidative stress such as Alzheimer's disease (AD), breast cancer, ischemic-reperfusion, cardiovascular disease, neurodegenerative disease, diabetes, HIV infection, arthrosclerosis, rheumatoid arthritis, thalassemia and including non human disease or ageing (80-87). The end products from oxidative stress such as malondialdehyde (MDA) from lipid oxidation, carbonyl content from protein oxidation, DNA fragmentation, free radical molecules and other by-products from metabolism were observed in many studies.

The ROS are also generated in thalassemia. Oxidant injury by ROS in thalassemia is one of their pathophysiology caused by the excess of unpaired globin chains (α- or β-chain) and unstable hemoglobin destruction to release heme, free iron,

and hemichrome (88). Iron overload was promoted oxidative stress by Fenton's reaction (8, 11). The hydroxyl radical or superoxide was generated by unstable hemoglobin. Oxidative stress was observed in thalassemia by Livrea *et al.*(8). Reactive oxygen species tend to increase the antioxidant enzyme activity in thalassemia patient over than healthy control (8, 16, 44, 45, 89). Excessive of free radicals lead chain reaction on lipid membrane including protein oxidation in thalassemic red blood cell (12).

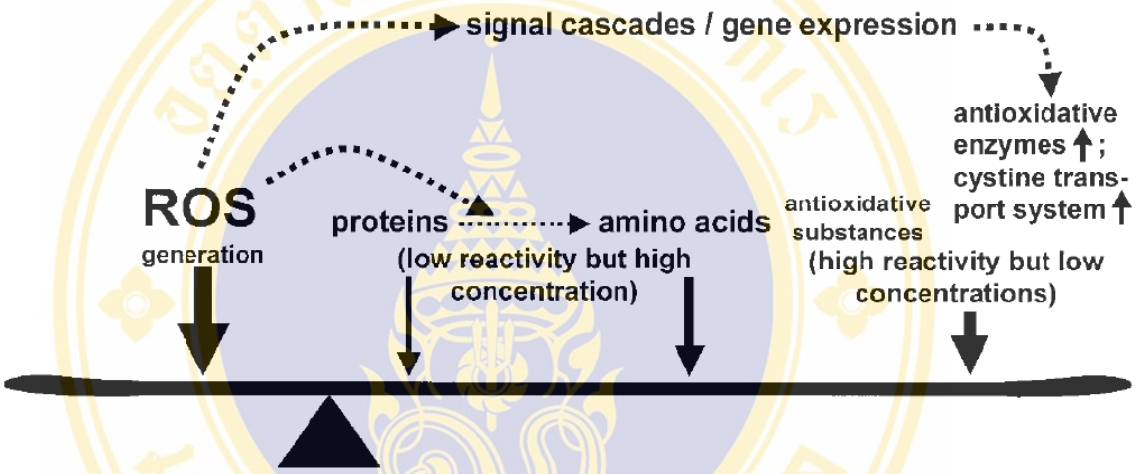

Figure 9 Mechanism of redox homeostasis (79)

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7. Protein oxidation

One of important molecules affected from oxidative injury is protein. It is a molecule that serves in cell structure, cell signaling, and enzyme process. The ROS can induce the oxidation on amino acid residue side chains, formation of protein-protein cross linkages and oxidation of protein backbone resulting in protein fragmentation (22, 90). The high level of ROS can oxidize nucleic acids, lipids, and protein. DNA and RNA without repair mechanism can lead to transcription and translation errors, causing oxidized protein (86, 91). Moreover, protein oxidation was initiated by many parameters such as peroxynitrite, nitric oxide and hypochlorite (11). The study of oxidation on bovine serum albumin by radiolysis was given the information about protein degradation, protein fragmentation and the oxidation system leads to an increase level of oxidized proteins (92).

The oxidative stress on protein is caused by directly attacking of ROS, such as hydroxyl radical ($OH^{\bullet}$) which attack directly to carbon-center of protein. The oxidation by attacking at the backbone protein was lead to generate hydroxyl protein derivative. The alkoxyl radical from protein oxidation was cleaved by diamide or α-amidation pathway to produce CO_2, NH_3, carboxylic acid and α-ketocarboxylic acid. The ionizing radiation or $OH^{\bullet}$ radical can also attack various amino acid side chains. The products from oxidation amino acid side chain are shown in Table 2. The metal-binding site of the enzyme is susceptible for ferrous ions binding and form ferrous iron-complexes which react with hydrogen peroxide and generate hydroxyl or hydroxyl-like free radicals (11, 86, 93, 94).

7.1 Protein carbonyl

The oxidation of protein by attacking of radical lead to protein cleavage either by α-amidation and carbonyl derivative are generated by direct attack of the radical. Carbonyl (C=O) groups are produced on protein side chains (especially of proline (Pro), arginine (Arg), lysine (Lys) and threonine (Thr)) when they are oxidized as shown in Table 2 (21, 22, 86). The presence of carbonyl groups has been used as a biomarker for protein oxidation which associates with ageing, oxidative stress and diseases. Carbonyl is the functional groups in aldehyde and ketone (95). It was found in organic compound and often encountered in biomolecules and play a crucial role in three-dimensional intermolecular associations (96). The protein

carbonyl derivative was produced via lipid peroxidation and glycoxidation during oxidative stress. Lipid membrane is commonly cleaved by oxidative radical and produced aldehydes such as malondialdehyde, hexanol, acrolein and 4-hydroxy-2-nonenal (HNE). The glycoxidation is the reaction of glucose and oxidants which produced methyl glyoxal and glyoxal (48, 97). The reactive carbonyl derivatives such as ketoamines, ketoaldehyde was generated by reaction of reducing sugar or protein oxidation on lysine residues (22). The formation of protein carbonyl during oxidative stress was observed in many human diseases such as Alzheimer's disease, chronic lung disease, chronic renal failure, diabetes and sepsis (98). Commonly, the marker of protein oxidation is a protein carbonyl. The generation of oxidized protein are stable than lipid peroxidation products, stable in serum and circulate longer period in blood (99). In the presence of carbonyl can further react with the α-amino on lysine residues and lead to protein cross-links. An aggregation of protein carbonyl cross-link is unable to degrade by protein degradation mechanism and lead to inhibit proteolytic process. The accumulation of oxidized protein can enhance cellular dysfunction (91).

7.2 Accumulation of oxidizing protein

The rate of protein oxidation and protein degradation results in accumulation of oxidized protein. As in Figure 10, the physiological environment and biological oxidation during respiratory in aerobic respiration promote the generation of radical species including ROS. The ROS formation can generate oxidized protein in the cell. Carbonyl compound is one of the important forms of oxidized protein because it is an active form and leads to protein cross-link. Oxidized protein normally degrades to amino acids by proteolytic system via proteosome and lysosome pathway (100). The reactive species and non-radical molecule were eliminated by protein and enzymes such as ferritin, transferrin, SOD, CAT, GPX. When the antioxidant activity becomes insufficient, ROS can also generate other chain reactions such as hydroxyl radical formation as product. The concentration of vitamins (C, E, A) and other metabolite such as bililubin and uric acid can scavenge free radicals. The accumulation of oxidized protein not only depends on rate of protein oxidation but also rate of degradation. Important process to degrade protein is proteolytic systems which eliminate oxidized and damage protein. Proteosome and lysosomal pathway is an essential pathway in the cells. Oxidized protein tends to be a sufficient target for

degradation by proteosome. Ubiquitin is a small protein and attach itself on oxidizied protein targeting to recognize by proteosome. The recycling by enzyme isopeptidase is used to disassemble ubiquitin back to active form. The short peptide chain can be hydrolyzed by other cellular peptidase. Furthermore, lysosome system degrades protein in both extracellular and intracellular. Lysosome engulfs the damaged protein with specific stimuli to maintain cellular homeostasis (100). Although, the proteolytic system is able to degrade oxidized or modified protein in the cell but there are some inhibition process occuring under oxidative stress. The products from oxidation and protein modification by glycolysis can inhibit protease activity. Moreover, in the oxidation of protein and protein cross-link from carbonyl derivative cannot degrade and also inhibits the protease enzyme. The unable repair of oxidized protein leads to protein accumulation in the cell (100).

Table 2 Protein oxidation products

Amino acid residue	Products formed
Arginine	Glutamic semialdehyde
Cysteine	Disulfides: Cys-S-S-Cys, Cys-S-S-R
Glutamate	4-Hydroxy-glutamate, Pyruvate, α-Ketoglutarate
Histidine	2-Oxo-histidine
Leucine	3-and 4-Hydroxy-leucine
Lysine	2-Aminoadipic-semialdehyde, 3-,4-, and 5-hydroxy-lysine
Methionine	Methionine sulfoxide, Methionine sulfone
Phenylalanine	2-, 3-, and 4-hydroxy-phenylanine
Proline	Glutamic semialdehyde, Pyroglutamic acid, 2-Pyrrolidone 4-hydroxy-proline
Threonine	2-Amino-3-keto-butyric acid
Tryptophan	N-Formyl-kynurenine, kynurenine, 2- ,4- ,5-, 6-, and 7-Hydroxy-tryptophan
Tyrosine	3-4-dihydroxy phenylanine (DOPA), Tyr-Tyr cross-linked proteins, 3-Nitro-tyrosine, 3,5-Dichloro-tyrosine
Valine	3-, 4–Hydroxy-valine

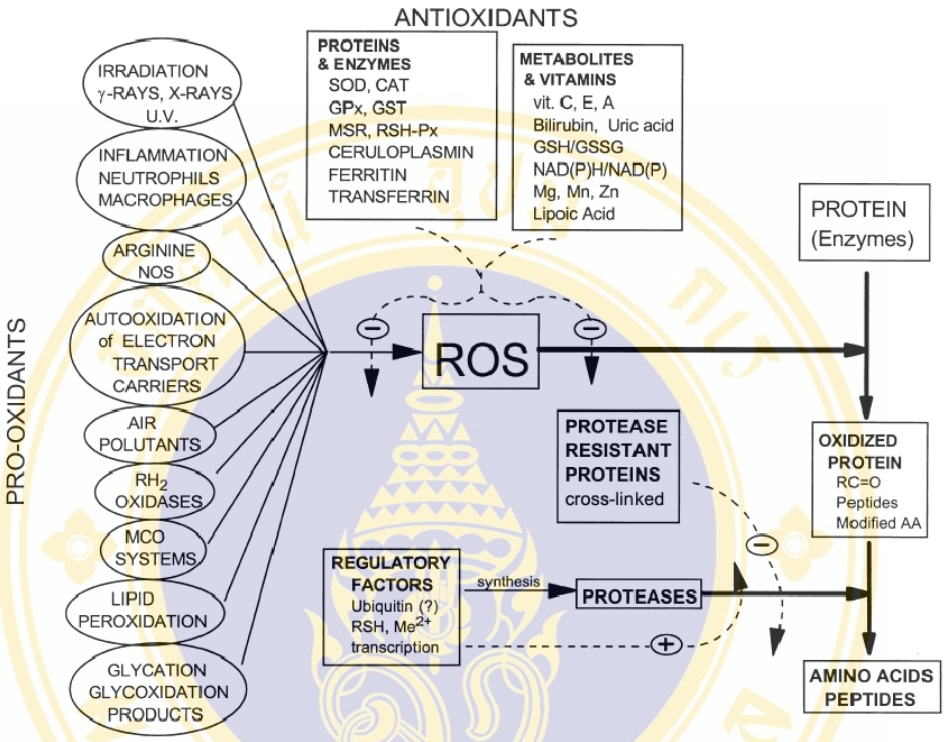


Figure 10 Protein oxidation and degradation (91)

8. Antioxidants

The protection of cell and organ system to deleterious effects of free radicals and ROS, exposure to free radicals from a variety of source has led the cell to develop the defense mechanisms against free radical-inducible oxidative stress. The antioxidant process such as prevent mechanism, repair mechanism, physical defenses, and antioxidant defenses had composed of enzymatic and non-enzymatic antioxidant (48). Enzymatic antioxidants; the first level defense against free radical damage are enzymes which are synthesized in the human body such as superoxide dismutase, glutathione peroxidase, and catalase. Superoxide dismutase (SOD) is isoenzyme containing copper, manganese or iron (CuSOD, MgSOD or FeSOD. Superoxide dismutase scavenging initiating free radical such as superoxide radical ($O_2^{\bullet -}$) or hydrogen peroxide (H_2O_2) (11). Catalase (CAT) contains four protein subunits, each of which contains a heme group within the active site. The active site is stabilized by one molecule tightly bound NADPH. CAT can decompose hydrogen peroxide to water and dioxygen. Glutathione peroxidase is a selenoenzyme which catalyzes lipid hydroperoxide (48). Non-enzymatic antioxidants are α-tocopherol (vitamin E), ascorbic acid (vitamin C), glutathione (GSH), carotenoids, and flavonoids. Dietary vitamins directly scavenge ROS and upregulates the activity of antioxidant system. Vitamin E is a lipid soluble phenolic derivative; it has a very active hydroxyl group. Vitamin E inhibits ROS-induced generation of lipid peroxyl radicals (48). Vitamin C has ability to reduce ferric to ferrous, the catalyst of the Fenton reaction. Vitamin C can remove hypochlorous acid (HOCl) by oxidation and convert to dehydroascorbic acid which is unstable molecule and break down to oxalic and threonic acid. The mechanism utilizes glutathione for converting dehydroascorbate back to ascorbic acid by enzyme dehydroascorbate reductase (48). Glutathione has an important role in protection against free radical damage which involves several enzymatic pathways.

9. Glutathione

Glutathione (γ-glutamyl-L-cysteinylglycine, GSH) is virtually presented in all animal cells. It is a tripeptide composed of cysteine, glutamic acid and glycine (Figure 11). Glutathione is produced in all organs, especially in liver. Glutathione can be free or bound to protein form which presents mostly in reduced form. In oxidative stress, it can be converted to the oxidized form (GSSG) during free radical scavenging. In normal condition, glutathione is present about 0.5-10 mM in mammalian cells whereas micromolar concentration is typically found in plasma. The sulfhydryl (-SH) group in cysteine residue is represented as an active group of glutathione. Glutathione involves in many cellular reactions such as scavenge free radical or ROS, reacting with physiological metabolite or xenobiotic compounds or conjugating with nitric oxide (101).

9.1 Glutathione biosynthesis and utilization

glutathione is synthesized in the cell by the sequential actions of γ-glutamylcysteine synthetase (GCS) and glutathione synthetase (GS) in six enzyme reactions (γ-glutamyl cycle) (Figure 10) (102). This pathway occurs in all cell types, glutathione mainly produced and exported by liver. Glutathione contains two characteristic groups that are γ-glutamyl linkage and sulfhydyl group. The synthesis of glutathione occurs in the cell by using enzymatically controlled reaction via ATP and non-essential amino acid as substrates.

The first step, a combination between cysteine and glutamate to form γ-glutamyl cysteine use enzyme GCS and ATP (reaction a, Figure 12a). In this step, GCS activity is inhibited by glutathione concentration; therefore it is a rate limiting step. Cysteine and glutamic acid is a non-essential amino acid. Cysteine is obtained from dietary protein, generated from methionine and endogenous protein turnover.

The second synthesis step is the addition of glycine by ATP dependent enzyme glutathione synthetase to γ-glutamyl cysteine dipeptide (reaction b, Figure 12b) and forming to γ-glutamyl-cysteinyl-glycine or glutathione (103). γ-Glutamylcysteine synthetase is heterodimer consisting of heavy (catalytic) subunit (GCSh, 73 kDa) and light (regulatory) subunit (GCSl, 30 kDa), the latter increasing the affinity of the heavy subunit for its substrate glutamate (102, 104, 105).

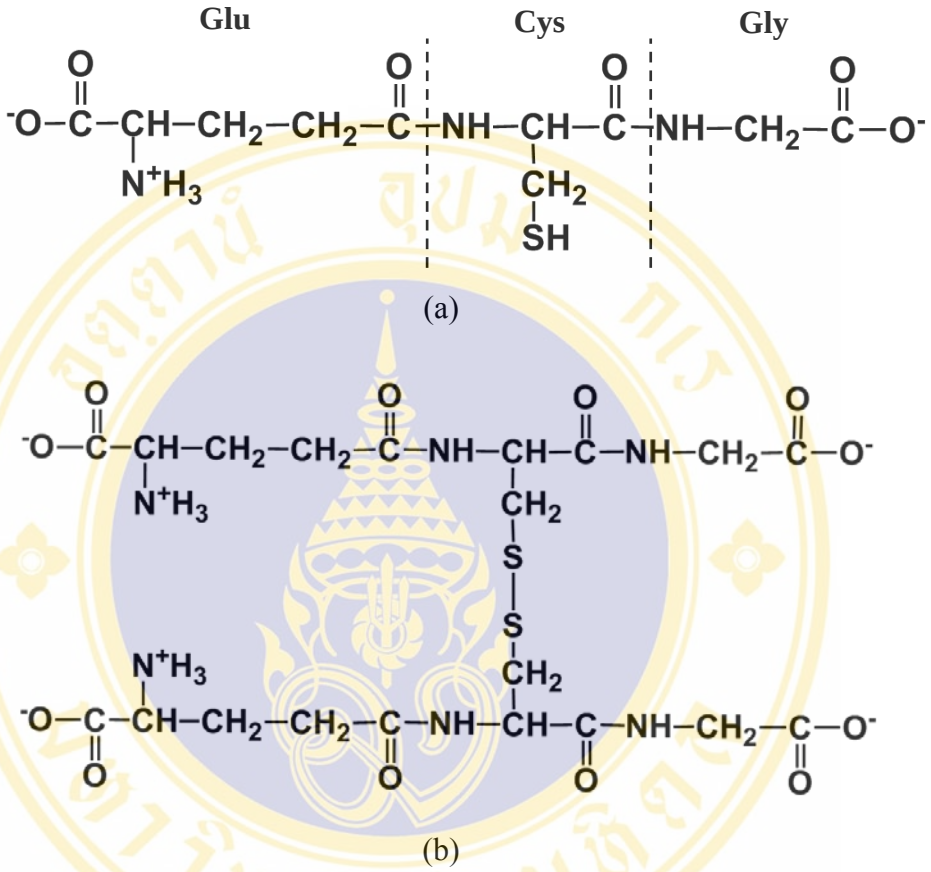


Figure 11 Glutathione structure (31, 106); (a) glutathione reduced form, (b) glutathione oxidized form

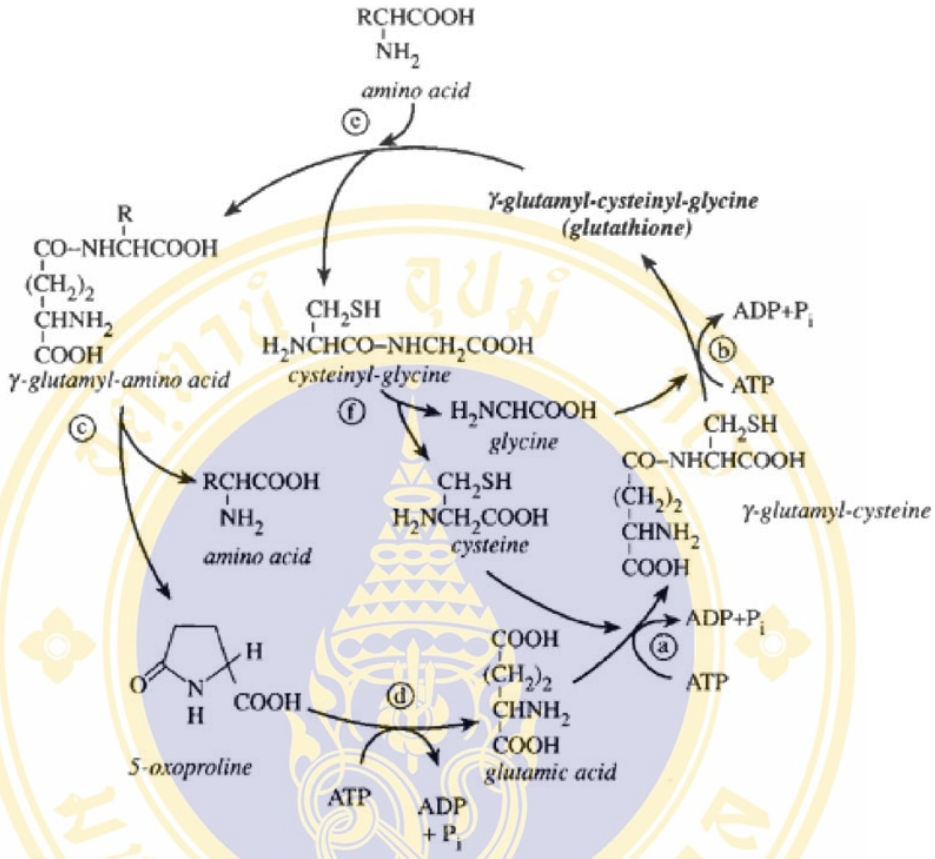


Figure 12 γ-Glutamyl cycle, (a) glutamylcysteine synthetase; (b) glutathione synthetase; (c) γ-glutamyl cyclotransferase; (d) oxoprolinase; (e) γ-glutamyl transpeptidase; (f) peptidase (18, 102)

Reaction a: L-cysteine + L-glutamate + ATP $\xrightarrow{\gamma\text{-glutamylcysteine synthetase}}$ L-γ-glutamyl-L-cysteine + ADP + Pi

Reaction b: L-γ-glutamyl-L-cysteine + glycine + ATP $\xrightarrow{glutathione\ synthetase}$ glutathione + ADP + Pi

Reaction c: glutathione $\xrightarrow{\gamma\text{-flutamyl transpeptidase}}$ γ-glutamyl + cysteinyl-glycine

Reaction d: cysteinyl-glycine $\xrightarrow{peptidase}$ glycine + cysteine

Reaction e: γ-glutamyl + free amino acid $\longrightarrow$ γ-glutamyl-amino acid

Reaction f: γ-glutmyl-amino acid $\xrightarrow{\gamma\text{-glutamyl cyclotransferase}}$ amino acid + 5-oxoproline

Reaction g: 5-oxoproline+MgATP $\xrightarrow{oxoprolinase}$ glutamate

In contrast to GSH synthesis which occurs intracellularly, GSH degradation occurs exclusively in the extracellular space, and only on the surface of cells that express the γ-glutamyl transpeptidase. Transportation of glutathione is regulated by enzyme γ-glutamyl transpeptidase. This enzyme is bound to the outer surface of cell membrane. Utilization of glutathione caused by action of γ-glutamyl transpeptidase enzyme leads to formation of cysteinyl-glycine and γ-glutamyl-amino acid (reaction c, F12c). Normally, plasma glutathione was in micromolar which is supplied by liver. γ-glutamyl transpeptidase also transports plasma glutathione into the cell (102). Cysteinyl-glycine is cleaved to cysteine and glycine by enzyme peptidase (reaction d, Figure 10f) and produce glutathione again in γ-glutamyl cycle. Free amino acid conjugate to glutathione by transpeptidation reaction to produce γ-glutamyl-amino acid (reaction e, Figure 12e). The γ-glutatmyl-amino acid is then converted by intracellular enzyme γ-glutamyl cyclotransferase to release free amino acid and produce 5-oxoproline (reaction f, Figure 12c). The 5-oxoproline is converted to yield glutamate by ATP dependent enzyme oxoprolinase (reaction g, Figure 12d).

Glutathione resists intracellular degradation and can only be metabolized extracellularly through the γ-glutamyl transpeptidase and γ-glutamyl cyclotransferase. The γ-carboxyl linkage at the terminal end of glutamate bonds to cysteine residue in glutathione. This is an unusual arrangement to make it resists to degradation by intracellular peptidases and can be hydrolyzed by γ-glutamyl transpeptidase (on the external surface). At the terminal end of glutathione, the glycine moiety is preferred to protect molecule from intracellular γ-glutamyl cyclotransferase which affects only with extracellular enzyme. As an action of amino acid linkage in glutathione structure tends to make it metabolized at extracellular level (104).

Normally, glutathione produced in the cell by using dietary amino acid as a substrate. Furthermore the hepatocyte cells can convert methionine to cysteine by transsulfuration pathway to produce glutathione (Figure 13). This pathway is very limited on tissue distribution which is observed in liver, kidney, intestine and pancreases. It is an irreversible process in conversion of methionine to cysteine. Methionnine is first converted to S-adenosylhomocysteine by enzyme methionine adenosyltransferase and ATP. The removal of adenosine is modulated by

hydrolase enzyme to form homocysteine. Homocysteine combine with serine to produce cystathione. The cleavage of cystathione brings to release cysteine which is used in glutathione biosynthesis. In this pathway, the conversion of homocystenine is an irreversible process (104, 107, 108).

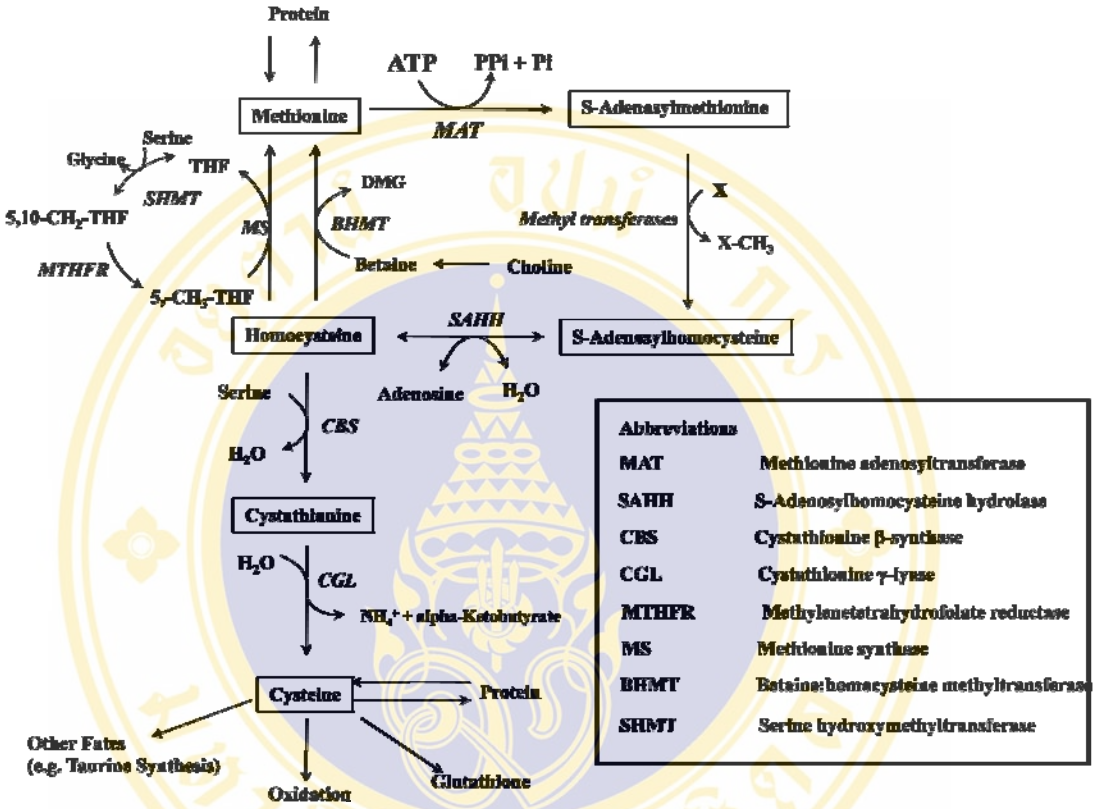


Figure 13 Glutathione synthesis by transfuration pathway (104)

9.2 Glutathione transport

After GSH synthesis, it is delivered to other intracellular compartments, including mitochondria and endoplasmic reticulum, and to the extracellular space (e.g., blood plasma and bile) for utilization by other cells and tissues. The transport of GSH through membrane is regulated by GSH transporter. Intracellular level of GSH in mammalian cells is in milimolar range, whereas micromolar is typically found in blood plasma. Glutathione itself is not significant transported into most of the cell but it broken down by transpeptidase enzyme that bound to outer surface. The breakdown products transport into the cell for systhesis GSH (Figure 14). The blood plasma is supported by liver. The inter-organ GSH transport was studied on liver and kidney. The hepatic vein plasma has a level of GSH higher than arterial blood plasma (102). The hepatic cells have high transpeptidase levels that utilize plasma GSH to form γ-glutamyl amino acids. In kidney, plasma glutathione is removed from plasma by transpeptidase enzyme (109). Plasma glutathione disappears rapidly in relation to GSH translocation. The transpeptidase inhibitor such as L-γ-glutamyl-(*o*-carboxy)-phenylhydrazide was used to inhibit the activity of γ-glutamyl transpeptidase, it leads to increase plasma glutathione levels (110-114). The major of transport form is GSH. γ-Glutamyl amino acids is formed during GSH transport out the cell by membrane bound transpeptidase, which concerns with recovery of amino acid constituents of GSH. Plasma GSH is also oxidized to GSSG by enzymatic oxidation (115). The accumulation of 5-oxoproline or γ-glutamyl cysteine in body fluid and excreted via urine is found in glutathione synthetase deficiency patients (105). Erythrocytes have high concentration of GSH which use to prevent hemoglobin oxidation. During oxidative stress GSH is converted to GSSG in erythrocyte cells. The active transport of GSH-conjugates and GSSG is linked to specific membrane ATPase.

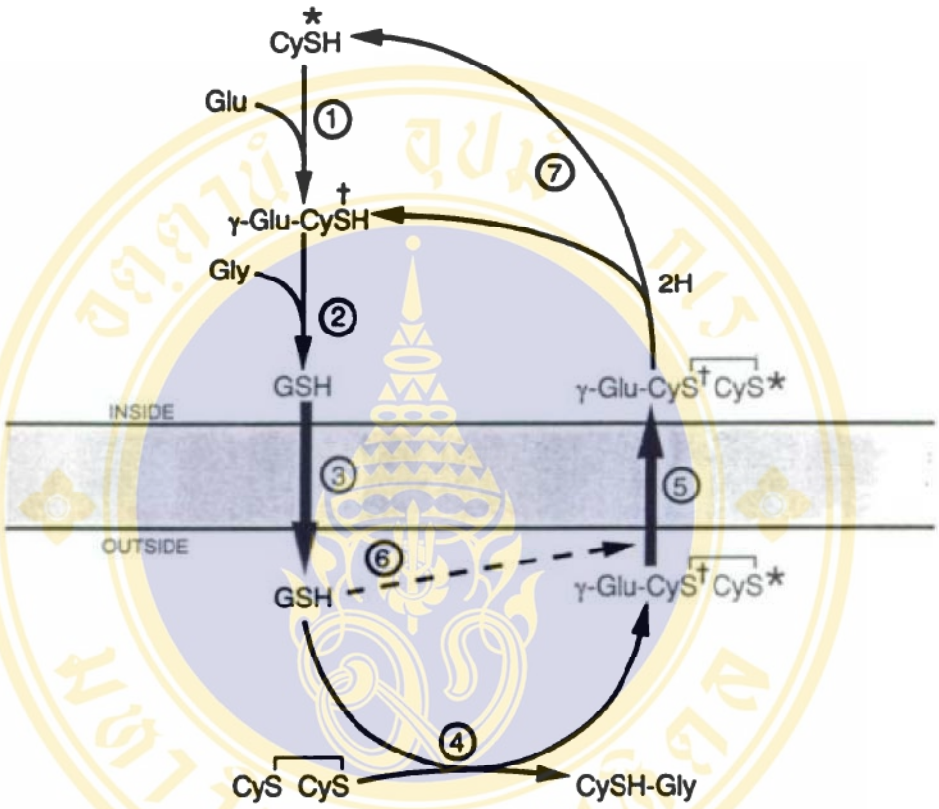


Figure 14 Salvage pathway of Glutathione (116). Glutathione (GSH), synthesized intracellularly (reactions 1 and 2), is exported (3) and reacts with y-glutamyl transpeptidase and extracellular cystine (4) to produce y-glutamylcystine, which is transported (5) and reduced intracellularly (7) to form cysteine and y-glutamylcysteine. Transport of y-glutamylcystine is inhibited by extracellular GSH (6).

9.3 Glutathione homeostasis and metabolism

The level of glutathione depends on the steady state of synthesis and degradation. The GSH synthesis is regulated by level of enzyme GCS and GS. The feedback mechanism is regulated by intracellular GSH (102). The low level of glutathione can be found in patients with hereditary γ-glutamylcysteine synthetase deficiency which is an autosomal recessive trait (117, 118).

The hepatocyte maintains GSH at a very high (8-10 mM) intracellular level (102, 108, 109, 119). Intracellular glutathione concentrations are maintained by two processes. First, it is regulated by ATP-direct synthesis (γ-glutamyl cysteninyl synthetase and glutathione synthetase). Secondly, GSSG recycle to GSH by enzyme glutathione reductase (GR) (Figure 15). Glutathione S-transferase (GST) is interesting to pharmacologists and toxicologists because they provide targets for antiasthmatic and antitumor drug therapies. The enzyme GST is probably the most important enzyme in drug metabolism which provide the conjugation GSH and electrophilic substances to generate mecapturates (stable and non-toxic) (120).

The contribution of ROS in the cells is disrupting by antioxidants. Glutathione serve as an antioxidant in the cells. Intracellular GSH is converted to GSSG by glutathione peroxidase during H_2O_2 reduction. The accumulation of GSSG during oxidative stress is recycling back to GSH by glutathione reductase (Figure 16).

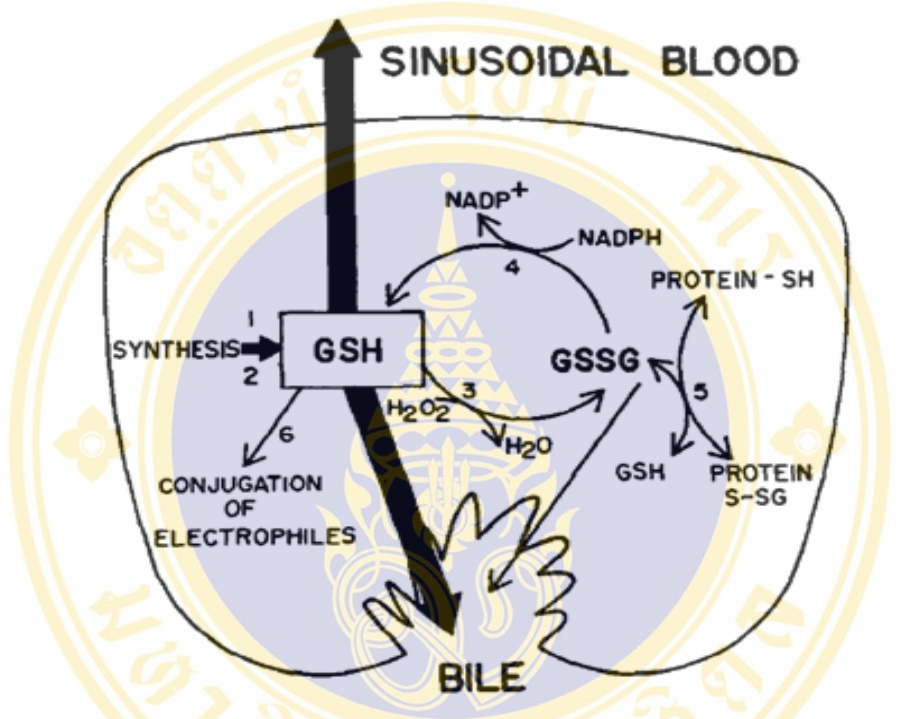


Figure 15 Hepatocyte glutathione synthesis (119). Reaction 1: γ-glutamylcysteine synthetase; reaction 2: GSHs ynthetase; reaction 3: GSH peroxidase; reaction 4: GSSG reductace; reaction 5: thioltransferase; reaction 6: GSH S-transferase. This model does not show the mitochondrial pool, which seems to have the same enzymes and distinct GSH regulation.

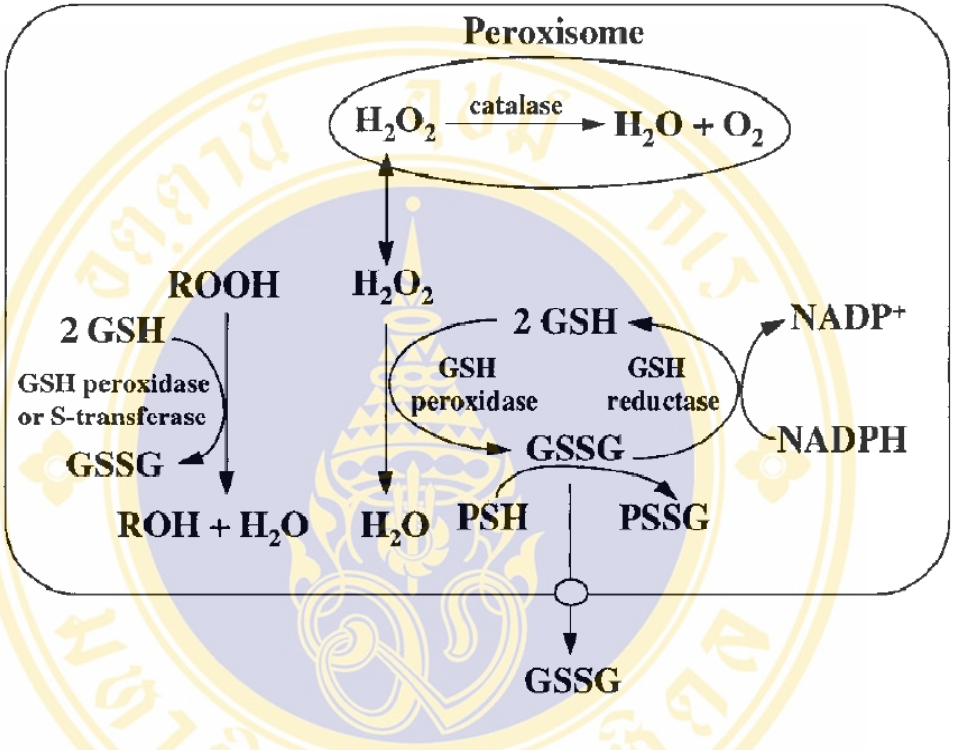


Figure 16 Antioxidant function of GSH (104)

9.4 Glutathione redox cycle

The conversion between reduced and oxidized form of glutathione is operated by glutathione related enzyme. Under normal physiological condition, glutathione is presented mainly in reduced form (GSH). During oxidative stress, the excessive of ROS is produced and damage cell. Reduced glutathione is a substrate of enzyme glutathione S-transferase and glutathione peroxidase for protect cells from oxidative damage. Oxidized glutathione (GSSG) is produced during this step. The accumulation of GSSG is able to reverse back to GSH by enzyme glutathione reductase. The ratio between GSH and GSSG is known as redox ratio. A redox ratio is depended on the oxidative stress condition and NADPH/$NADP^+$ pool. Furthermore, the ratio of GSH/GSSG was used a critical determinant in the cells (34). The rise of GSH/GSSG ratio is observed in diabetes patients which become a major role in glucose homeostasis in diabetes (121). A redox ratio of GSH/GSSG is used as a redox state in diseases.

9.5 Glutathione S-transferase

Glutathione S-transferase or also known as glutathione transferase has commonly abbreviation as GST. This enzyme is contributed to the metabolism of drugs, pesticides, other xenobiotics and peroxide products from oxidative stress. Glutathione S-transferase can be divided into three groups: canonical (orcytosolic) GSTs (cGSTs), mitochondrial GSTs and microsomal GSTs (120). In human, it can be divided into several classes such as GST alpha, Mu, Pi, Theta, Omega class (122), recently Zeta (123). These enzymes catalyze nonpolar electrophilic carbon compound, nitrogen or sulfur atom to non toxic molecules by attaching with GSH. Their substrates are include halogenonitrobenzenes, arene oxides, quinones, and α-, β-unsaturated carbonyls. Glutathione S-transferase has been considering role in phase II of drug metabolism. The functional group offers as an electrophilic center that attack by GSH during xenobiotic or metabolite metabolism.

The ability of GSTs is inactivating potential cytotoxic and genobiotic compounds. This is an important role in the detoxification of a board spectrum of chemical that leads to cytotoxicity or mutagenic compounds. The conjugation of GSH and ultimate carcinogen compound such as aflatoxin B1 produce a harmless metabolite that readily to eliminate from the cell. Other compounds such as pesticides

or dichlorodiphenyltrichloroethane (DDT) can metabolize by GST. Endogenous compounds are also formed as by-products of normal metabolism that able to catalyze by GST. The production of ROS such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^\bullet$) are from aerobic respiration. The oxidation of lipid membrane by free radical leads to membrane destruction. In addition, other radicals are produce during lipid oxidation and lead to chain reaction. The aerobic metabolites are able to catalyze by GST, which reduce them to harmless molecule. Furthermore, GST can reduce DNA hydroperoxide such as thymine peroxide (124). Glutathione S-transferase catalyzes the general reaction shown as:

$$GSH + R\text{-}X \rightarrow GSR + HX$$

Xenobiotic compounds are eliminated by GST through mercapturic acid pathway. The conjugation between glutathione and xenobiotic is excreted immediately in bile and transport to kidney. The conjugation of GSH and xenobitic are transport out the cells by active transport. Glutathione is split to γ-glutamyl by enzyme γ-glutamyl transpetidase, glycine by dipeptidase, and cysteine. The cysteine is excreted as a mercapturic acid, which is one of earlier xenobiotic metabolite compound (123). The other break-down products are used to generate GSH via γ-glutamyl cycle.

The oxidation on nucleotides produces adenine propenal and hydroperoxide that catalyze by GST (120) (Figure 17). Oxidative stress products such as $O_2^{\bullet-}$, H_2O_2 and $HO^\bullet$ can damage lipid membrane. Lipid hydroperoxide is produced during lipid oxidation during lipid oxidation. The enzyme GSTs provide a conjugation of GSH and various lipid hydroperoxide products such as cholesteryl hydroperoxides or fatty acid hydroperoxides.

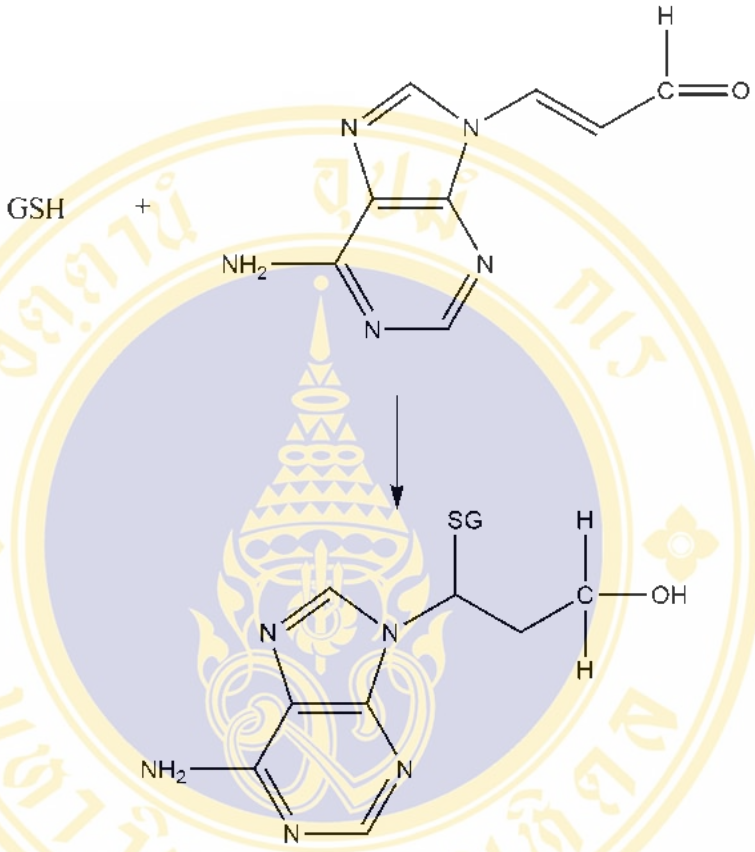


Figure 17 Reaction catalyzed by GST on substrate (adenine propenal) (120)

9.6 Glutathione peroxidase

The glutathione peroxidase (GPX) catalyzes degradation of organic hydroperoxides by reduction, as two glutathione molecules (represented as GSH) are oxidized to a disulfide. The H_2O_2 is catalyzed by GPX by using GSH as a substrate. Glutathione peroxidase uses the trace element selenium as functional group. The selenium is an essential atom in the active site. The enzyme-bound selenium can undergo a substrate-induced redox change (125).

$$H_2O_2 + 2GSH \xrightarrow{glutathione\ peroxidase} H_2O + GSSG$$

$$ROOH + 2GSH \xrightarrow{glutathione\ peroxidase} ROH + H_2O + GSSG$$

This enzyme presents as four types: (1) classical GPX (GPX-1); (2) gastrointestinal GPX (GPX-GI); (3) plasma GPX (GPX-P); and (4) phospholipids hydroperoxide GPX (PHGPX) (126). It has an important role in mitochondrial whereas catalase is absence. The reduced form of enzyme contain selenol (-SeH) at the active site. The reduction of hydrogen peroxide was following the elementary step.

$$(E\text{-}SeH) + H_2O_2 \rightarrow (E\text{-}SeOH) + H_2O \quad .....(1)$$

$$(E\text{-}SeOH) + GSH \rightarrow (E\text{-}Se\text{-}SG) + HOH \quad .....(2)$$

$$(E\text{-}Se\text{-}SG) + GSH \rightarrow (E\text{-}SeH) + GS\text{-}SG \quad .....(3)$$

$$H_2O_2 + 2GSH \rightarrow GS\text{-}SG + 2H_2O \quad .....(4)$$

The selenoate (Se-) in active site of GPX molecule convert to selenenic acid (SeOH) in the first step of reaction. The addition of one GSH molecule, selenenic acid is transformed to a selenenylsulfide adducts. The second molecule of GSH is used to regenerate the active selenoate. The oxidized glutathione was form during hydrogen peroxide or lipid hydroperoxide by using GPX (127). In this reaction is usually coupled with glutathione redox cycle, the enzyme glutathione reductase subsequently revert GSSG back to GSH for maintain GSH level in the cell.

9.7 Glutathione reductase

Glutathione reductase (GR) is required for maintainance of GSH during oxidative stress. It is a homodimeric flavoprotein, each subunit is essential for catalytic site. This enzyme is usually presented as an essential for maintenance of reduced glutathione levels in *vivo*. The nicotinamide adenine dinucleotide phosphate (NADPH) is used during reduction of GSSG in glutathione redox cycle which catalyze by glutathione reductase (128).

$$GSSG + NADPH + H^{+} \longrightarrow 2GSH + NADP^{+}$$

NADPH is produced in the cell via pentose phosphate pathway. The ribose-5-phosphate (Ru5P) is an essential precursor. The oxidation of glucose-6-phophate to Ru5P by using glucose-6-phophate dehydrogenase (G6PD), which yields NADPH from $NADP^{+}$ (31). NADPH, itself scavenges the radical derive from peroxynitrite involved in many inflammatory diseases. The inactive radical is caused by the attacking of NADPH in one electron step that can convert to anion and easily to protonate. NADPH is served as hydrogen donor such as coenzyme or antioxidant activity. Other role of NADPH is an indirect antioxidant. It contributes to antioxidant antioxidant potential of the cell by regenerating the oxidized antioxidant. As a coenzyme ($NADPH+H^{+}$) of glutathione reductase, it donates a hydrogen atom to GSSG recycle back to GSH (129).

9.8 Function of glutathione

Glutathione is found in free or bound protein form. The free form is mainly present in reduced glutathione (GSH), which is converted to oxidized form (GSSG) during oxidative stress by glutathione peroxidase and reverse to reduced form by glutathione reductase (18). Glutathione is participated in many cellular reactions. The major role of glutathione is protection of the cell from oxidative stress. It has an ability of antioxidant activity and also serves as coenzyme in enzymatic antioxidant. The reducing power of glutathione is participated in many cellular antioxidant mechanisms.

9.8.1 Glutathione scavenge free radicals or ROSs

Glutathione has a very important role in protective against free radical damage by providing the equivalents for several enzymes (Figure 16). The antioxidant enzyme GST and GPX is used GSH as a substrate to catalyze the lipid hydroperoxide. Furthermore, GPX is also used to catalyze the reduction of H_2O_2 and couple with action of GR to maintain a level of GSH. The oxidized form of glutathione is generated by his step and reversed back to reduced form by glutathione reductase.

Reduced glutathione (GSH) scavenge a radical such as hydroxyl and produce the thiyl radical ($RS^{\bullet}$) (Table 2). Thiyl radicals participate in many reactions such as electron transport, hydrogen abstraction and other reaction in detoxification of xenobiotics (130). The thiyl radical from GSH is converted to GSSG by glutaredoxin (131). GSH react with superoxide anion ($O_2^{\bullet-}$) lead to generate the $^1O_2$ (48) or $O_2^{\bullet-}$ (11). The accumulation of GSSG occurs during free radical or ROS scavenge. Lipid membrane peroxidation predominately presents in oxidative stress and produce MDA as a marker for detection. Glutathione is used by GPX to catalyze lipid hydroperoxide (LOOH) and convert to LOH, GSSG and water molecule in protecting process from oxidative injury (48). Various kinds of oxidative products are neutralized by activity of enzyme whereas the other products are directly attached to GSH. Hydroxyl radical is directly added to double bond of DNA result in DNA damage. Thinly radical is formed during GSH scavenges the DNA radical (table 2) (132), (133).

Table 3 Glutathione scavenge free radicals or ROS

	Reaction	Equation
1	Hydroxyl radical quenching	$GSH + HO^{\bullet} \rightarrow GS^{\bullet} + H_2O$
2	Secondary radical quenching	$GSH + R^{\bullet} \rightarrow GS^{\bullet} + RH$
3	Quenching of radical center on DNA	$GSH + DNA^{\bullet} \rightarrow GS^{\bullet} + DNA$
4	Quenching of DNA peroxyl radicals	$GSH + DNAOO^{\bullet} \rightarrow GS^{\bullet} + DNAOOH$
5	Reduction of lipid peroxides, catalyzed GPX	$2GSH + LOOH \rightarrow GSSG + LOH + H_2O$
6	Maintenance of protein –SH groups in the reduced state	$2GSH + PSSX \rightarrow GSSG + P(SH)2X$
7	Recycling of vitamin C from its oxidized radical	$2GSH + 2Asc^{\bullet} \rightarrow GSSG + 2Asc$
8	Conjugation with P450 products, catalyzed by GST	$GSH + Substr^{\bullet} \rightarrow$ Substr-GS complex

9.8.2 Glutathione detoxification reaction

The endogenous or exogenous compound is able to conjugate with GSH. The interaction of foreign compounds with GSH is catalyzed by GST. The conjugation between glutathione and foreign compounds are converted to mercapturic acids. The exogenous compounds such as heavy metals, drug metabolites or other xenobiotics are harmful to biological molecules, which neutralized by GSH and convert to less harmful. The foreign compound that neutralize by GSH is excreted into urine or bile acid. The endogenous metabolites such as products from unstable ketosteroid in human liver protein conjugate with GSH (102). Furthermore, enzyme GST concerning with drug metabolism process in human liver cells to eliminate drug metabolite in hepatocyte cells. Reduced glutathione has loss hydrogen atom and produce GS-conjugate with metabolite in drug metabolism phase II. The catalyzing effect accelerate by GST enzyme activity (122). The conjugation products are exported out the cell by ATPase activity such as in erythrocytes cell. The transport of glutathione conjugated products are modulated by multidrug resistance-associated protein or pump out the cell by GSSG-Mg^{2+} ATPase (134).

9.8.3 Glutathione reserve function of vitamins

The other role of glutathione is the regeneration of vitamin E or vitamin C back to their active form. After oxidation of vitamins, they change to radical form and this form is inactive. This radical of vitamin can reduce back to active form by GSH (116, 135). Dehydroascorbic acid is an oxidation product during ascorbic acid (vitamin C) catalyzes ROS molecules. The lack of ability to synthesize ascorbic acid in human, this ascorbic acid is restoring to active form by using GSH and enzyme dehydroascorbate reductase (48).

9.8.4 Glutathione as a coenzyme

There are many enzymes are influenced by GSH. The oxidation of lipid membrane by attacking of free radical such as $OH^{\bullet}$ lead to hydroperoxide (LOOH) formation (136). Lipid hydroperoxides are reduced to alcohol and water by enzyme glutathione peroxidase using GSH as electron donor (50). Glutathione has mainly protected role against oxidative stress to act as cofactor for several detoxifying enzyme, e.g. GPX and GST (137). The GSH acts as a substrate for the enzyme during catalytic reaction of hydrogen peroxide, lipid hydroperoxides

and electrophilic compounds. The enzyme activity leads to lower level of total intracellular glutathione. The aggregation of GSSG in the cell can be released to maintain intracellular GSH/GSSG ratio. Oxidized glutathione was degraded extracellular to increase the cellular requirement for GSH synthesis. Glutathione disulfide can be also reverted back to GSH by glutathione reductase, which used NADPH as a reductant (101).

CHAPTER III
MATERIALS AND METHODS

1. Subjects

Subjects were divided into 2 groups:

1.1 Normal subjects

Thirty normal volunteers, male and female, ages ranging from 18 to 50 years, with normal hematological data and hemoglobin typing were recruited. All subjects were healthy on clinical assessment and signed informed consents. This trial was approved by The Ethic Committee on Research involving human subject, Faculty of Medicine Siriraj Hospital, Mahidol University.

Inclusion criteria for normal subjects were as follows:

1. no history of all types of allergy.
2. not use of any drug during the past month.
3. no smoke and drink alcohol.
4. no pregnant and lactation.
5. signed in informed consents.

1.2 β-thalassemia/Hb E patients

Thirty patients with hemoglobin typing of β-thalassemia/Hb E disease and age between 18-50 years were recruited. Subjects did not receive blood transfusion and no drug consumption or iron chelator medication at least 3 months prior study.

Inclusion criteria for β-thalassemia/Hb E patients were as follows:

1. hemoglobin level between 6-9 g/ dl.
2. aspartate aminotransferase or alanine aminotransferase ≤ 3 x upper limit of normal.
3. creatine clearance ≥ 10 ml/min.
4. written informed consents.

Exclusion criteria β-thalassemia/Hb E patients were as follows:

1. pregnancy or breastfeeding.
2. blood transfusion or iron chelator medication within 3 months before study.

β-thalassemia/Hb E patients were selected from the project "curcumin as an antioxidant in thalassemia", which approved by The Ethic Committee on Research involving human subject, Faculty of Medicine Siriraj Hospital, Mahidol University. Blood samples were collected before curcumin administration, using 1 ml of whole blood and 500 µl of plasma.

2. Blood collection

Five milliliters of blood were collected into vacutainer tubes containing ethylenediamine tetrachloroacetic acid (EDTA) from normal volunteers. Whole blood was centrifuged at 800 x g for 10 minutes. Plasma and buffy coat were removed. RBC pellets were washed three times with phosphate buffered saline (154 mM NaCl and 10 mM phosphate buffer, pH 7.4) by centrifuged at 830 x g for 5 minutes to precipitate RBC. Red cell sample was mixed with same buffer to make 50% red cell suspension. RBC and plasma were kept at -80 ^{o}C. The red cell suspension and plasma were aliquoted for various hematological and biochemical parameter analysis.

3. Biochemical parameters

Oxidative status and antioxidant were measured. The oxidative status was composed of level of total glutathione, GSH, GSSG, redox ratio (GSH/GSSG) and plasma protein carbonyl. Antioxidant enzyme activities were glutathione S-transferase (GST), glutathione reductase (GR) and glutathione peroxidase (GPX).

4. Chemicals

Table 4 Chemicals used in this study

No	Name of chemicals	Formula	M.W.	Trade Mark
1	Potassium phosphate dibasic	K_2HPO_4	174.18	Sigma
2	Potassium phosphate monobasic	KH_2PO_4	136.09	Sigma
3	1-chloro-2,4-dinitrobenzene (CDNB)	$C_6H_3ClN_2O_4$	202.55	Fluka
4	Glutathione reduced (GSH)	$C_{10}H_{17}N_3O_6S$	307.32	Sigma
5	β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH)	$C_{21}H_{26}N_7Na_4O_{17}P_3$	833.35	Sigma
6	Glutathione oxidized (GSSG)	$C_{20}H_{32}N_6O_{12}S_2$	612.63	Sigma
7	Sodium phosphate dibasic heptahydrate	$Na_2HPO_4.7H_2O$	268.07	Sigma
8	Sodium phosphate monobasic monohydrate	$NaH_2PO_4.H_2O$	137.99	Sigma
9	Tris (hydroxymethyl) aminomethane	$NH_2C(CH_2OH)_3$	121.14	Sigma
10	Disodium ethylenediamine tetrachloroacetic acid (EDTA)	$C_{10}H_{14}N_2O_8$ $Na_2H.2H_2O$	372.20	Sigma
11	Glutathione reductase from baker's yeast			Sigma
12	tert-Butyl hydroperoxide (TBHP)	$(CH_3)_3COOH$	90.12	Sigma
13	Sulfosalicylic acid dihydrate	$C_7H_6O_6S.\ 2H_2O$	254.21	Sigma
14	Ethanol	CH_3CH_2OH	46.07	Sigma
15	Hydrochloric acid	HCl	36.46	Sigma
16	Sodium borohydride	$NaBH_4$	37.83	Sigma
17	Dithiothreitol	$C_4H_{10}O_2S_2$	154.25	Sigma
18	Bromobimane	$C_{10}H_{11}BrN_2O_2$	271.11	Sigma
19	Cysteamine	$NH_2CH_2CH_2SH$	77.15	Fluka
20	4-Ethylmorpholine	$C_6H_{13}NO$	115.17	Fluka

Table 4 Chemicals used in this study (continued)

No	Name of chemicals	Formula	M.W.	Trade Mark
21	N-ethylmaleimide	$C_6H_7NO_2$	125.13	Sigma
22	1-Octanol	$CH_3(CH_2)_7OH$	130.23	Sigma
23	Ammonium nitrate	NH_4NO_3	80.04	Sigma
24	Ammonium formate	CH_5NO_2	63.06	Fluka
25	Methanol	CH_3OH	32.04	J.T. Baker
26	Acetonitrile	CH_3CN	41.05	J.T. Baker

5. Instruments

5.1 Vortex, Genie 2, USA

5.2 Autopipette pipetman 2, 20, 100, 200 and 1000, Gilson, France

5.3 Analytical balance, Balance Scaltec, model BC52, Germany

5.4 Analytical balance, Sartorius scientific, model R160P, Germany

5.5 Magnetic stirrer, Magnestir, Lab-Line Instrument Inc., USA

5.6 pH meter, Model RL150, Russell, USA

5.7 Refrigerated centrifuge, Sorvall RC 26 plus, rotor SS-34, Dupont, USA

5.8 Refrigerated centrifuge, Sorvall RT 7, Dupont, USA

5.9 Spectrophotometer, UV-160, Shimadzu, Japan

5.10 Spectrophotometer, UV-1601, Shimadzu, Japan

5.11 Waterbath, Precision scientific, Illinois, USA

5.12 Ultra-Low Temperature Chest Freezers, Sanyo, Japan

5.13 Ultra-Low Temperature Chest Freezers, Ultima II, Revco, USA

5.14 Alliance HPLC 2695 separation module, Waters, USA

5.15 Waters 2475 Multi Wavelength Fluorescence Detector, Waters, USA

5.16 Synergy HT Multi-detection microplate reader, BioTek Instruments, USA

5.17 Column ODS hypersil dimension150 x 4.6 mm, particle size 5 µ with Hypersil BDS C18 column guard, Thermo Electron Corporation, USA

6. Glassware and miscellaneous

6.1 Disposable sterile non-pyrogenic needle 21G and 22G gauge needle, Terumo Corporation, Japan

6.2 Disposable sterile non-toxic non-pyrogenic syringe 10 ml, 20 ml, Terumo Corporation, Japan

6.3 VACUETTE® EDTA tubes 9 ml and 3 ml, Greiner bio-one, Austria.

6.4 Beaker

6.5 Volumetric flask

6.6 Parafilm, 4 inch x 125 ft roll, size M, American nation, USA

6.7 Pipette tip 2, 200, and 1000 µl

6.8 Pasteur pipette

6.9 Suction volumetric flask

6.10 Eppendorf microtube 1.5, 2 ml

6.11 Glass tube 3 ml

6.12 Glass vial, Waters, USA

7. Buffer and reagent preparation

7.1 Buffer for preparation of red blood cell

Phosphate buffered saline 5 mM, pH 7.4 was prepared by 1.0616 g of $Na_2HPO_4.7H_2O$ 0.1435 g of $NaH_2PO_4.H_2O$ and NaCl 8.5 g were mixed with 800 ml of distilled water. The mixture was adjusted pH to 7.4 and volume to 1000 ml with distilled water. This solution was kept at 4°C and used within 3 months.

7.2 Buffer and reagent for GST determination

7.2.1 0.1 mM Phosphate buffer, pH 6.5

The buffer was prepared by mixing $KH_2PO_4.3H_2O$ 1.1104 g and K_2HPO_4 7.2888 g with 400 ml of distilled water and adjusted pH to 6.5. The distilled water was added to final volume of 1000 ml. It was stored at 4 °C and used within 3 months.

7.2.2 2 mM CDNB

1-chloro-2,4-dinitrobenzene 0.004051 g was dissolved in 1 ml of 95% ethanol. This solution was freshly prepared and kept at 4 ^{o}C.

7.2.3 5 mM GSH

GSH 0.01537 g was dissolved in 1 ml of cold-distilled water. This solution was freshly prepared for every test.

7.3 Buffer and reagent for GR determination

7.3.1 139 mM phosphate buffer

K_2HPO_4 7.3569 g and KH_2PO_4 3.7111 g were mixed and dissolved in distilled water 400 ml. This solution was adjusted pH to 7.4 and volume to 500 ml with distilled water.

7.3.2 2.5 mM NADPH

NADPH 0.0021 g was diluted in 1. 5 ml eppendorf microtube with 1 ml of ice-cold distilled water. This solution was freshly prepared and kept on ice until used.

7.3.3 22 mM GSSG

GSSG 0.0135 g was dissolved in 1 ml of ice-cold distilled water and kept on ice until used.

7.4 Buffer and reagent for GPX determination

7.4.1 1 M Tris-HCl, 5 mM EDTA, pH 8.0

Tris-HCl 12.1140 g and EDTA 0.1861 g were mixed with 80 ml of distilled water by magnetic stirrer. The solution was adjusted pH to 8.0 with concentrated hydrochloric acid and adjusted volume to 100 ml. This solution was kept at room temperature and used within 3 months.

7.4.2 2 mM NADPH

NADPH 0.0017 g was diluted with 1 ml of ice-cold distilled water, mixed it well and freshly prepared every experiment.

7.4.3 10 U/ml Glutathione reductase (GR)

Glutathione reductase 58.59 µl was added to 1441.41 µl of 1 M Tris-HCl, 5 mM EDTA, pH 8.0. This solution was prepared for 4 samples and should be prepared freshly before assay.

7.4.4 0.1 M GSH

GSH 0.0077 g was dissolved in 250 µl of distilled water in 1.5 ml eppendorf microtube. The solution was mixed by vortex. This solution was prepared for 4 samples and should be prepared freshly before assay.

7.4.5 7 mM *t*-Butyl hydroperoxide

One microliter of 70% *t*-Butyl hydroperoxide was added into distilled water in 1 ml volumetric flask and mixed. This solution should be prepared freshly before use.

7.5 Buffer and reagent for glutathione analysis

7.5.1 Buffer and reagent for blood glutathione extraction

7.5.1.1 10 mM phosphate buffer, pH 7.2

KH_2PO_4 0.1345 g and K_2HPO_4 0.1762 g were mixed by 100 ml HPLC grade water (MilliQ). This solution was adjusted pH to 7.2 and adjusted volume to 200 ml. This buffer was kept at 4 ^oC and used within 3 months.

7.5.1.2 10 mM phosphate buffer/0.1 M *N*-ethylmaleimide, pH 7.2

N-ethylmaleimide (NEM) 0.00063 g was mixed with 500 µl of 10 mM phosphate buffer (pH 7.2) in 1.5 ml eppendorf microtube mixed by vortex. This solution was freshly prepared in every experiment.

7.5.1.3 12% sulfosalicylic acid (w/v)

Sulfosalicylic acid 12 g was dissolved by 100 ml HPLC-grade water and kept at 4 ^oC. This solution was used within 3 months.

7.5.2 Reagent for standard preparation

7.5.2.1 0.1 M HCl/10 µM DTT

12 N HCl 1.67 ml mixed with HPLC grade water 198.33 ml. After mixing, 0.0003 g of DTT was added. This solution was kept at 4 ^oC and used within 3 months.

7.5.2.2 Glutathione standard (20-100 µM)

Glutathione 0.0031 g was dissolved in ice-cold HPLC grade water 1 ml in 1.5 ml eppendorf microtube. The solution was mixed by vortexing and used as a stock solution of 10 mM GSH. The stock solution of GSH 2 µl was mixed with 10 mM HCl/DTT 998 µl for preparing 20 µM GSH. The other

concentration was 40, 60, 80 and 100 µM were prepared by mixing the stock solution 4, 6, 8 and 10 µl with 10 mM HCl/DTT 996, 994, 992, and 990 µl, respectively. This standard was freshly prepared and always kept on ice.

7.5.2.3 Cysteamine standard

5 mM cysteinamine was freshly prepared as a stock solution by mixing 0.0004 g cysteamine with 1 ml of ice-cold HPLC grade water. The 50 µM cysteamine was prepared by mixing 10 µl cysteamine stock solutions with 990 µl of ice-cold HPLC grade water in eppendorf microtube 1.5 ml. This 50 µM cysteamine standard was freshly prepared before used.

7.5.3 Reagent for derivatization

7.5.3.1 2 mM EDTA/DTT

EDTA 0.0074 g and DTT 0.0031 g were mixed with 80 µl HPLC grade water in beaker and adjusted volume to 100 ml in volumetric flask. This solution was kept at 4 ^{o}C and used within 3 months.

7.5.3.2 4 M $NaBH_4$

$NaBH_4$ 0.1513 g was dissolved in 1 ml of a solution of 333 ml dimethyl sulfoxide and 66 mM NaOH. This solution was mixed by vortex and kept at 4 ^{o}C. It was freshly prepared before used.

7.5.3.3 1.8 M HCl

12 N HCl 37.5 ml was diluted with 212.5 ml HPLC grade water, at 4 ^{o}C, and used within 3 months.

7.5.3.4 25 mM bromobimane

Bromobimane 0.0068 g was dissolved in 1 ml of acetonitrile in 1.5 ml eppendorf microtube covered with foil. The solution was yellow and must be kept in the dark. This was stored at -20 ^{o}C and stable for a week.

7.5.4 Reagent for chromatography

30 mM Ammonium nitrate and 40 mM ammonium formate buffer, pH 3.6 were used to equilibrate the column. It was prepared by dissolving 2.4012 g ammonium nitrate and 2.5224 g ammonium formate with 800 ml HPLC grade water and stirred. The solution was adjusted pH to 3.6 and volume to 1 liter by volumetric flask. This buffer was kept at 4 ^{o}C for 3 months. Reagent should be let stand at room temperature before used.

8. Buffer and reagents for protein carbonyl

Protein carbonyl content was measured by using protein carbonyl assay kit (Cayman Chemical Company, USA). All reagents were prepared according to the assay kit's manual.

8.1 Hydrochloric acid

12 M HCl was slowly added to 40 ml HPLC-grade water to yield 2. 5 M HCl. The diluted HCl was stable for at least 3 months at room temperature.

8.2 2,4-Dinitrophenylhydrazine (DNPH)

DNPH was dissolved by 10 ml of 2.5 M HCl. Reconstituted DNPH was stable for 1 week at 4^{o}C and stored in the dark.

8.3 TCA solution

Trichloroacetic acid (TCA) solution in the vial contained 2 g/ml. The 12 ml of TCA solution was slowly added to 108 ml of HPLC-grade water. This dilution resulted in a 20% TCA solution. 40 ml of this solution was diluted with 40 ml of HPLC grade water to make 10% TCA. This solution was stable for one week at room temperature.

8.4 Ethyl acetate and ethanol

The ethyl acetate and ethanol were mixed for 1:1 ratio.

9. Sample analysis

9.1 Hematological parameters

Complete blood count (CBC) was determined in 2 ml of the EDTA blood by automated cell counter. Number of white cells, red blood cells, hemoglobin concentration, percent hematocrit, MCV, MCH, MCHC, platelet counts, and reticulocytes count were recorded in both whole blood and 50% red cell suspension. Hemoglobin typing was also analyzed by Department of Hematology, Faculty of Medicine Siriraj hospital.

9.2 Glutathione assay

Glutathione used as an antioxidant in the cell involving 3 enzymes: glutathione S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPX).

9.2.1 Assay of glutathione S-transferase activity

Principle

Total GST activity (cytosolic and microsomal) was measured by the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione (138).

$$GSH + CDNB \xrightarrow{\textit{glutathione S-transferase}} GS\text{-}CDNB + Cl^- + H^+$$

The conjugation is accompanied by an increase in absorbance at 340 nm for 5 minutes. The rate of increase is directly proportional to the GST activity in the sample.

Procedure

The red cell suspension was diluted to 1:100 by ice-cold distilled water and placed on ice. The 768 µl of 0.1 M phosphate buffer, pH 6.5 was mixed with 100 µl of 0.5 mM GSH in 1.5 ml eppendorf and then add 100 µl of blood sample. The reaction was started by adding 32 µl of 2 mM CDNB in 95 % ethanol (the concentration of ethanol in the final assay will be maintained below 5%). The control without blood sample contained 868 µl of 0.1 M phosphate buffer, pH 6.5 was mixed with 0.5 of 100 µl of mM GSH and 32 µl of 2 mM CDNB. The activity of the enzyme expressed as 1 µmol/min/mg hemoglobin (U/mg Hb) with extinction coefficient 0.0096 $\mu M^{-1}cm^{-1}$ in specific activity by correcting for Hb protein content.

Calculation:

The enzyme activity was expressed as unit per mg Hb and was calculated as:

$$\text{GST activity} = \frac{\Delta Abs340/min}{0.\ 0096\ \mu M} \times \frac{V_t}{V_s} \times (d.f.)\ U/ml$$

$$\text{GST specific activity} = \frac{\text{GST activity U/ml}}{\text{Hb mg/ml}}$$

where

V_t = total volume

V_s = sample volume

d.f. = dilution factor

9.2.2 Assay of glutathione reductase

Principle

Glutathione reductase activity was based on the oxidation of NADPH to NADP, which catalyzed by a limiting concentration of GR (139).

$$GSSG + NADPH + H^+ \xrightarrow{glutathione\ reductase} 2GSH + NADP^+$$

The enzyme activity was quantified by the change in absorbance at 340 nm using extinction coefficient for NADPH 6.22 $mM^{-1}cm^{-1}$. The rate of decrease in the A340 was directly proportional to the GR activity in the sample.

Procedure

The activity of GR was determined in red cell suspension by oxidation reaction. The blood samples were diluted to 1:50 by iced-cold distilled water. The solution of 940 µl of 139 mM phosphate buffer, 0.76 mM EDTA, pH 7.4 was mixed with 20 µl of 22 mM GSSG in 1.5 ml eppendorf microtube and then added 20 µl of blood sample. The reaction of enzyme activity was started by adding of 20 µl of 2.5 mM NADPH. The control without blood sample contained 960 µl of 139 mM phosphate buffer, 0.76 mM EDTA, pH 7.4 was mixed with 20 µl of 22 mM GSSG and 20 µl of 2.5 mM NADPH in 1.5 ml eppendorf. The activity was measured by using 40 sec for Lag time and monitored for 60 sec.

Calculation:

Glutathione reductase activity was dependent on the rate of decreasing of absorbance at 340 nm. The reaction rate at 340 nm can be determined by using the NADPH extinction coefficient of 0.00622 $\mu M^{-1}cm^{-1}$. The enzyme activity was expressed as unit per Hb mg.

$$\text{GR activity} = \frac{\Delta\text{Abs340/min}}{0.00622\ \mu\text{M}} \times \frac{V_t}{V_s} \times (\text{d.f.})\ \text{U/ml}$$

$$\text{GR specific activity} = \frac{\text{GR activity U/ml}}{\text{Hb mg/ml}}$$

where

V_t = total volume

V_s = sample volume

d.f. = dilution factor

9.2.3 Assay of glutathione peroxidase

Principle

Glutathione peroxidase catalyzes the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) by hydroperoxide, where ROOH is a hydroperoxide. The *t*-butyl hydroperoxide is the most suitable substrate for assay of the enzyme. The rate of formation of GSSG is measured by the oxidation of NADPH using glutathione reductase (GR) reaction.

$$2GSH + R\text{-}O\text{-}O\text{-}H \xrightarrow{\textit{glutathione peroxidase}} R\text{-}O\text{-}H + GSSG + H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow{\textit{glutathione reductase}} 2GSH + NADP^+$$

The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. The rate of decrease in the A340 is directly proportional to the GPX activity in the sample

Procedure

20 µl of red cell suspension was diluted into 180 µl of distilled water and frozen at –80°C for 15 minutes and then thawed. 670 µl of distilled water (as reagent blank) or 660 µl of distilled water (for unknown sample) and 100 µl of 1M Tris-HCl, 5 mM EDTA, pH 8.0 were mixed in 1.5 ml eppendorf microtube. After mixing water and Tris-HCl by vortexing, the following reagents were then added, 100 µl of 2 mM NADPH, 100 µl of 10 U/ml GR, 10 µl hemolysate (1:10) and 0.1 M GSH. The mixture was immediately incubated at 37°C, for 10

minutes. Finally, after incubation, 10 µl of 7 mM *t*-butyl hydroperoxide was added into unknown sample (not in reagent blank) and measured at 340 nm for 2 minutes.

Calculation:

All reagents should be prepared fresh and carried on ice. The rate of the reaction depends on the concentration of *t*-butyl hydroperoxide. The GPX activity was expressed as international units per gram of Hb and was calculated with extinction coefficient 6.22 $mM^{-1}cm^{-1}$ as:

$$\text{GPX activity} = \frac{100 \times \Delta OD_{340} \times V_c}{Hb \times 6.22 \times V_H} \text{ U/g Hb}$$

where

V_c = total volume (1 ml)

Hb = hemoglobin concentration (1:10 g/dl)

V_H = volume of Hb (10 µl)

9.3 Measurement of glutathione level

Principle

High-performance liquid chromatography (HPLC) is a recently method of choice for measuring glutathione. This technique is rapid, highly specific, sensitive, and reproducible.

The level of total glutathione and oxidized glutathione (GSSG) are determined by using specific and sensitive HPLC method with fluorometric detection as described by Pastore (140). The oxidation of thiol during sample extraction leads to overestimation of disulfide. The thiol group blocking agent is *N*-ethylmaleimide (NEM) which is added before blood extraction for oxidized glutathione. Blood protein is extracted by sulfosalicylic acid and removed by centrifugation.

The determination of glutathione in both plasma and blood sample requires the reduction of disulphide bond between glutathione and other thiols. To maintain thiol group in a reduced state, dithiothreitol and sodium borohydride ($NaBH_4$) were used. Octanol has been used to protect foam as a surface-reactive agent during reaction. The determination in a various type of glutathione is used a

fluorometric detection coupled with HPLC. Bromobimane has been used for determining reduced, oxidized, and protein-bound glutathione in plasma and whole blood. Bimanes bind rapidly with thiol at pH 8.0 at room temperature to produce a highly fluorescent thioether. Fluorescence bimane adduct after derivatization can detect in fluorescent detector during elution through specific column.

Procedure

Whole blood extraction

After collecting blood, 100 µl of whole blood with EDTA was added in each 2 eppendorf microtube and then mixed immediately with 12 µl of 10 mM phosphate buffer, pH7.2 (for total GSH and GSH) or with 12 µl of 10 mM phosphate buffer and 0.1 M NEM, pH7.2 (for GSSG). The mixture of this solution was hemolyzed by adding 900 µl ice-cold distilled water and deproteinized by adding 200 µl of salfosalicylic acid (12% w/v). This mixture was centrifuged at 10,000 x g, 5 minutes and the glutathione in the supernatant (acid-soluble fraction) was determined.

Glutathione derivatization:

The derivertized solution was prepared before adding the sample. 10 µl of 2 mM EDTA and DTT and 15 µl of 4 M $NaBH_4$ were added in the new eppendorf microtube. The 5 µl of 1-octanol (for prevent foaming during reaction), 10 µl of 1.8 M HCl were then added. After mixing, 50 µl of internal standard (50 µM cysteinamine) and 50 µl of extracted supernatant sample were added. After mixture was incubated for 3 minutes, 50 µl of 1.5 M *N*-ethylmorpholine buffer (pH 8.0), 40 µl of ice-cold ultrapure water and 10 µl of 25 mM bromobimane were added. The mixtures were then incubated for another 3 minutes, 20 µl of acetic acid was finally added. The 20 µl of this mixture was injected into column.

Chromatography

The derivertized sample was injected into a 150 x 4.6 mm Hypersil-ODS column equilibrated with 30 mM ammonium nitrate and 40 mM ammonium formate buffer, pH 3.6 (buffer A). Acetonitrile was used as a buffer B, a 6-min gradient of acetonitrile (0-4 min, 0-30% buffer B; 4-5 min, 30-100% buffer B; 5-6 min, 100% buffer B) used to separate the thiol at flow rate 1.5 ml/min. The fluorescence detector was operated at an excitation wavelength of 390 nm and an emission wavelength 478 nm. The column was equilibrated for 10 min and run at

ambient temperature.

Linearity of glutathione

Calibration curve for glutathione was prepared in duplicate by diluting the stock solution with 0.1 M HCl and 100 μM DTT to make 20, 40, 60, 80 and 100 μM of glutathione. The linearity of this assay was obtained from 0-100 μM glutathione. The limit of detection was defined as the concentration that makes a signal-to-noise ratio >5 was about 50 nM.

Recovery, accuracy and precision of glutathione

Known concentration of GSH at 3 different concentrations (150, 250, and 500 μM) were added to whole blood sample. The blood samples with glutathione addition were determined in 6 replicates. The intra-assay or accuracy was obtained by analyzing the GSH in blood sample at the same day with 8 different times.

Calculation of glutathione

Calibration curve and level of glutathione was calculated by using Empower software®. Total glutathione was obtained from the sample without NEM and the oxidized glutathione was obtained from sample with NEM. The reduced glutathione was obtained from subtraction of total glutathione and oxidized glutathione.

All of the reagents for HPLC were prepared by using a HPLC grade. HPLC grade water was usually used to dissolve the reagent and to hemolyze. Blood sample used for analyzing glutathione must be kept on ice and analyzed on the same day. Otherwise, samples can be stored at –80 ^{o}C for a week.

9.4 Measurement of protein carbonyl level

Principle

Reactive oxygen species can generate highly reactive molecule that caused a chain reaction. Hydrogen peroxide or other radical can transform amine group on amino acid side-chain (i.e. lysine, arginine, proline, or histidine) into carbonyls. The protein carbonyl contents can also be used as a biomarker of protein oxidation. The most common procedure is the reaction between protein carbonyls group and 2,4-dinitrophenylbenzene (DNPH). The reaction between carbonyl and DNPH forms a shift base to produce hydrazone, which can be determined by spectrophotometer.

Procedure

Plasma sample 200 µl was added into two of 2 ml eppendorf microtube, one for sample and another for a blank. 800 µl DNPH was added to the sample tube and 2.5 M HCl to the blank tube. After the addition of DNPH or HCl, both tubes were incubated in the dark at room temperature for 1 hour and mixed by vortex every 15 minutes. After incubation, 1 ml of 20% TCA was added to each tube and mixed. They were let stand on ice for 5 minutes before centrifuged at 10,000 x g for 10 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 1 ml of 10% TCA then follow the procedure as same as 20% TCA adding step. After centrifugation, the supernatant was discarded. The pellet was resuspended in 1 ml of 1:1 ethyl acetate and ethanol solution. Pellet was suspended by spatula, mixed, and centrifuged at 10,000 x g for 10 minutes at 4°C. This pellet was washed by ethyl acetate and ethanol for 3 times. After final wash, the pellet was resuspended in 500 µl of guanidine hydrochloride by vortex. It was then centrifuged at 10,000 x g for 10 minutes at 4°C. The 200 µl of supernatant was removed to 96-well plate to measure the protein carbonyl at wavelength between 360-385 nm by Synergy HT Multi-detection microplate reader.

Calculation:

The KC4® software for microplate reader was used to calculate the average absorbance of each well plate. The subtraction of absorbance between blank and sample was a corrected absorbance (CA). The extinction coefficient for DNPH at 370 nm is 0.011 µM-1 has been adjusted for the path length of the solution in the well.The protein carbonyl level was calculated by equation:

$$\text{Protein carbonyl } \left(\frac{\text{nmol}}{\text{ml}}\right) = \left(\frac{\text{CA}}{0.\ 011\mu\text{M}^{-1}}\right) \times \left(\frac{500\ \mu\text{l}}{200\ \mu\text{l}}\right)$$

10. Statistic analysis

Two-tailed non-parametric Mann-Whitney test was used for comparison between groups. A value of p-value<0.05 was considered statistically significant.

CHAPTER IV
RESULTS

1. Hematological data of normal subjects and β-thalassemia/Hb E patients

Hematological data of normal subjects were shown in Table 5 and values were mean ± SEM. Thirty normal subjects were 14 males and 16 females. The normal subjects had normal hematological typing (HbA_2 and HbA) and the mean value of hemoglobin was 14.26 ± 0.29 g/dl. The hemoglobin concentration was 15.44 ± 0.28 g/dl in males which was significantly higher than in females 13.23 ± 0.30 g/dl. The higher level of RBC, MCH, and platelets were also observed in males (Table 6).

Thirty β-thalassemia/Hb E patients were divided into 14 males and 16 females. There were 10 splenectomized (SP) patients (6 males and 4 females) and 20 non-splenectomized (NS) patients (8 males and 12 females) (Table 7). The mean value of hematological data of both groups of patients were shown as mean ± SEM in Table 8. The high value of MCV and MCH was found in males of SP patients group (p=0.037, 0.017) whereas it was low in NS patients (p=0.002, 0.002). The significant difference of WBC, MCV, MCH, platelet, and reticulocyte was observed in both of SP and NS patients (p=0.008, <0.001, 0.017, <0.001, and 0.023, respectively) (Table 9). The MCHC level in SP patients was lower than in NS patients (p=0.002). The mean value of hemoglobin in 30 β-thalassemia/Hb E patients was 7.07 ± 0.27 g/dl. The hemoglobin concentration observed in splenectomized (7.13 ± 0.62 g/dl) and non-splenectomized (7.03 ± 0.27 g/dl) were not significantly different, p=0.871 (Table 9).

The mean value of Hb, RBC, MCV, MCH, and MCHC in normal subjects were higher than patients whereas hct, WBC and reticulocyte were lower (p<0.001) but the platelet in both groups were comparable (p=0.535).

Table 5 Hematological data of normal subjects

Name	Sex	Age	Hematological data								
			Hb	Hct	WBC	RBC	MCV	MCH	MCHC	platelet	reticulocyte
			g/dl	%	10^3/ml	10^6/ml	fl	pg	g/dl	10^3/ml	%
CHT	M	24	17.00	14.10	6.63	5.86	83.60	29.00	34.70	258	0.98
SAI	M	38	16.10	14.00	5.11	5.29	87.50	30.40	34.80	221	1.37
NAT	M	20	15.40	12.70	4.47	5.17	88.40	29.80	33.70	240	1.24
PAI	M	24	15.50	13.30	5.80	4.98	89.00	31.00	35.00	211	1.60
PIS	M	24	16.30	13.20	6.50	5.30	90.00	31.00	34.00	210	1.00
KRT	M	24	15.40	13.40	4.40	5.11	88.00	30.00	34.00	221	0.60
ADS	M	26	15.50	13.10	6.10	4.97	90.00	31.00	35.00	195	0.90
CHP	M	49	13.60	12.20	5.90	4.90	85.00	28.00	33.00	206	1.60
PRH	M	22	15.90	13.00	7.46	5.43	85.30	29.30	34.30	237	0.94
AEP	M	23	16.10	18.20	4.80	5.22	93.00	31.00	33.00	244	0.70
TNS	M	24	15.90	14.60	10.10	5.14	92.00	31.00	34.00	250	1.20
KOM	M	24	13.30	14.00	5.20	4.41	89.10	30.20	33.80	247	1.03
SRV	M	24	15.80	14.30	7.90	5.21	91.00	30.00	33.00	226	0.90
WTC	M	24	14.30	13.30	4.97	5.00	89.00	29.00	32.00	275	0.90
Mean $\pm$ SEM	(n=14)		15.44 $\pm$ 0.28	13.81 $\pm$ 0.38	6.10 $\pm$ 0.42	5.14 $\pm$ 0.09	88.64 $\pm$ 0.71	30.05 $\pm$ 0.25	33.88 $\pm$ 0.24	231.50 $\pm$ 6.00	1.07 $\pm$ 0.08

Table 5 Hematological data of normal subjects (continued)

Name	Sex	Age	Hematological data								
			Hb g/dl	Hct %	WBC 10^3/ml	RBC 10^6/ml	MCV fl	MCH pg	MCHC g/dl	platelet 10^3/ml	reticulocyte %
RTY	F	40	13.90	13.10	5.30	4.87	85.40	28.50	33.40	312	1.18
SUP	F	29	11.50	13.70	6.99	3.92	91.30	29.30	32.10	267	1.16
PCG	F	24	12.40	13.90	5.59	4.28	87.10	29.00	33.20	332	2.35
AUK	F	36	12.10	12.40	3.82	4.48	83.90	27.00	32.20	184	1.17
PAN	F	24	13.40	12.70	5.39	5.04	84.30	26.60	31.50	249	0.60
KNT	F	24	12.70	13.30	4.10	4.49	86.20	28.30	32.80	234	0.89
BSP	F	26	12.50	14.70	6.70	4.15	91.00	30.10	33.10	329	1.01
WAP	F	50	13.00	13.20	5.05	4.31	90.50	30.20	33.30	251	1.42
SUG	F	22	15.10	13.50	8.56	5.00	88.60	3.20	34.10	268	1.37
PRA	F	39	13.60	13.90	9.96	4.50	89.10	30.20	33.90	347	1.66
ARP	F	24	13.60	13.10	6.40	4.68	89.00	29.00	33.00	179	0.70
SUK	F	22	15.10	13.70	8.20	5.01	89.00	30.00	34.00	247	1.40
SUC	F	23	13.80	16.60	6.43	4.83	84.70	28.60	33.70	318	0.86
PAP	F	24	11.60	13.60	4.15	3.98	91.00	29.10	32.00	274	0.77
SKN	F	24	15.10	13.70	8.20	5.01	89.00	30.00	34.00	247	1.40
TNP	F	35	12.20	15.70	6.60	4.17	91.00	29.00	32.00	190	1.40
Mean $\pm$ SEM	(n=16)		13.23 $\pm$ 0.30	13.80 $\pm$ 0.27	6.34 $\pm$ 0.44	4.55 $\pm$ 0.10	88.19 $\pm$ 0.65	27.38 $\pm$ 1.63	33.02 $\pm$ 0.21	264.25 $\pm$ 13.24	1.21 $\pm$ 0.11

Table 6 Comparison of hematological data in normal subjects between males and females

Subjects		Hematological data*								
		Hb g/dl	**Hct %**	**WBC 10^3/ml**	**RBC 10^6/ml**	**MCV fl**	**MCH pg**	**MCHC g/dl**	**platelet 10^3/ml**	**reticulocyte %**
Normal subjects										
Male	(n=14)	15.44 ± 0.28	13.81 ± 0.38	6.10 ± 0.42	5.14 ± 0.09	88.64 ± 0.71	30.05 ± 0.25	33.88 ± 0.24	231.50 ± 6.00	1.07 ± 0.08
Female	(n=16)	13.23 ± 0.30	13.80 ± 0.27	6.34 ± 0.44	4.55 ± 0.10	88.19 ± 0.65	27.38 ± 1.63	33.02 ± 0.21	264.25 ± 13.24	1.21 ± 0.11
p-value		0.001	0.975	0.692	<0.001	0.648	0.142	0.011	0.041	0.317
Total	(n=30)	14.26 ± 0.29	13.81 ± 0.22	6.23 ± 0.30	4.82 ± 0.85	88.40 ± 0.47	28.63 ± 0.90	33.42 ± 0.17	248.97 ± 8.06	1.14 ± 0.07

*Values were given as mean ± SEM

Table 7 Hematological data of β-thalassemia/Hb E patients

Name	Sex	Age	Type	Hematological data								
				Hb g/dl	**Hct** %	**WBC** 10^3/ml	**RBC** 10^6/ml	**MCV** fl	**MCH** pg	**MCHC** g/dl	**platelet** 10^3/ml	**reticulocyte** %
NPJ	M	42	SP	9.80	28.40	55.30	3.80	74.30	25.70	34.50	481	12.60
APR	M	25	SP	7.30	24.20	27.90	3.50	69.50	21.00	30.20	371	7.40
NPN	M	22	SP	6.50	21.20	244.00	2.70	79.10	24.30	30.70	702	8.50
PJJ	M	30	SP	5.60	18.30	66.40	2.50	72.00	22.00	30.60	814	38.00
GRJ	M	20	SP	6.10	20.80	58.20	3.30	63.40	18.60	29.30	800	-
TYT	M	28	SP	8.20	27.60	55.40	3.70	74.20	22.00	29.70	611	22.50
Mean ± SEM		(n=6)		7.25 ± 0.63	23.42 ± 1.64	84.53 ± 32.33	3.25 ± 0.22	72.08 ± 2.17	22.27 ± 1.02	30.83 ± 0.77	629.83 ± 72.48	17.80 ± 5.71
VPK	F	21	SP	4.70	17.30	86.40	2.80	62.00	16.80	27.20	879	12.60
PLP	F	17	SP	7.60	26.50	68.70	4.00	66.30	19.00	28.70	836	6.90
CTM	F	30	SP	5.00	18.50	54.20	2.70	69.00	19.00	27.60	779	4.50
APT	F	44	SP	10.50	32.40	31.30	5.80	55.90	18.10	32.40	320	4.50
Mean ± SEM		(n=4)		6.95 ± 1.35	23.68 ± 3.55	60.15 ± 11.65	3.83 ± 0.72	63.30 ± 2.86	18.23 ± 0.52	28.98 ± 1.18	703.50 ± 129.46	7.13 ± 1.91
Total		(n=10)		7.13 ± 0.62	23.52 ± 1.61	74.78 ± 19.56	3.48 ± 0.31	68.57 ± 2.17	20.65 ± 0.90	30.09 ± 0.69	659.30 ± 64.27	13.06 ± 3.63

Table 7 Hematological data of β-thalassemia/Hb E patients (continued)

Name	Sex	Age	Type	Hematological data								
				Hb g/dl	Hct %	WBC 10^3/ml	RBC 10^6/ml	MCV fl	MCH pg	MCHC g/dl	platelet 10^3/ml	reticulocyte %
CNB	M	45	NS	8.70	24.90	3.10	5.70	43.50	15.20	34.90	107	2.00
SRP	M	52	NS	8.50	25.60	15.20	4.80	52.90	17.60	33.20	308	3.50
VSB	M	16	NS	6.80	22.70	10.90	4.00	56.60	17.00	30.00	264	2.50
TSC	M	19	NS	6.20	18.90	7.90	3.50	53.80	17.70	32.80	360	2.70
DNN	M	18	NS	7.50	23.10	7.60	4.30	53.50	17.40	32.50	303	2.30
SPS	M	41	NS	6.30	19.30	5.60	4.00	48.70	15.90	32.60	139	4.20
TNK	M	39	NS	6.80	20.30	5.90	4.00	51.10	17.10	33.50	112	1.90
SYU	M	27	NS	6.10	19.80	5.60	3.90	51.30	15.80	30.80	155	2.90
Mean ± SEM		(n=8)		7.11 ± 0.36	21.83 ± 0.92	7.73 ± 1.33	4.28 ± 0.24	51.43 ± 1.40	16.71 ± 0.33	32.54 ± 0.54	218.50 ± 35.70	2.75 ± 0.28

Table 7 Hematological data of β-thalassemia/Hb E patients (continued)

Name	Sex	Age	Type	Hematological data								
				Hb g/dl	Hct %	WBC 10^3/ml	RBC 10^6/ml	MCV fl	MCH pg	MCHC g/dl	platelet 10^3/ml	reticulocyte %
CNP	F	22	NS	6.90	21.70	10.90	3.80	56.80	18.10	31.80	274	3.20
MNR	F	31	NS	7.90	23.00	9.80	3.90	59.40	20.40	34.30	110	2.80
YSN	F	54	NS	6.40	20.30	3.60	3.50	58.50	18.40	31.50	144	2.00
GST	F	30	NS	8.40	24.70	7.90	4.50	54.40	18.50	34.00	232	2.30
YPN	F	21	NS	6.80	21.60	9.60	3.80	57.00	17.90	31.50	153	5.30
JTJ	F	35	NS	6.00	19.70	5.50	3.00	66.30	20.20	30.50	117	3.50
BSP	F	49	NS	5.90	18.00	4.70	2.80	63.60	20.80	32.80	149	2.80
PGS	F	24	NS	9.10	27.50	7.70	3.70	73.50	24.30	33.10	245	2.10
APP	F	24	NS	9.40	27.60	7.00	4.70	58.80	20.00	34.10	268	1.70
PTN	F	28	NS	5.30	16.50	8.00	3.10	53.10	17.00	32.10	238	2.10
VTY	F	27	NS	6.00	17.60	5.70	3.10	56.40	19.20	34.10	221	1.00
VPJ	F	34	NS	5.70	19.80	23.20	3.10	63.70	18.30	28.80	201	6.60
Mean ± SEM		(n=12)		6.98 ± 0.40	21.50 ± 1.05	8.63 ± 1.47	3.58 ± 0.17	60.13 ± 1.66	19.43 ± 0.56	32.38 ± 0.49	196.00 ± 16.92	2.95 ± 0.46
Total		(n=20)		7.04 ± 0.27	21.63 ± 0.71	8.27 ± 1.01	3.86 ± 0.16	56.65 ± 1.48	18.34 ± 0.47	32.45 ± 0.35	205.003 ± 17.14	2.87 ± 0.29

Table 8 Comparison of hematological data in β-thalassemia/Hb E patients between males and females

Subjects		Hematological data*								
		Hb g/dl	**Hct** %	**WBC** 10^3/ml	**RBC** 10^6/ml	**MCV** fl	**MCH** pg	**MCHC** g/dl	**platelet** 10^3/ml	**reticulocyte** %
β-thalassemia/Hb E Splenectomized										
Male	n=6	7.25 ± 0.63	23.42 ± 1.64	84.53 ± 32.33	3.25 ± 0.22	72.08 ± 2.17	22.27 ± 1.02	30.83 ± 0.77	629.83 ± 72.48	17.80 ± 5.71
Female	n=4	6.95 ± 1.35	23.68 ± 3.55	60.15 ± 11.65	3.83 ± 0.72	63.30 ± 2.86	18.23 ± 0.52	28.98 ± 1.18	703.50 ± 129.46	7.13 ± 1.91
p-value		0.573	0.943	0.573	0.390	0.037	0.017	0.202	0.605	0.154
Total	n=10	7.13 ± 0.62	23.52 ± 1.61	74.78 ± 19.56	3.48 ± 0.31	68.57 ± 2.17	20.65 ± 0.90	30.09 ± 0.69	659.30 ± 64.27	13.06 ± 3.63
β-thalassemia/Hb E Non-splenectomized										
Male	n=8	7.11 ± 0.36	21.83 ± 0.92	7.73 ± 1.33	4.28 ± 0.24	51.43 ± 1.40	16.71 ± 0.33	32.54 ± 0.54	218.50 ± 35.70	2.75 ± 0.28
Female	n=12	6.98 ± 0.40	21.50 ± 1.05	8.63 ± 1.47	3.58 ± 0.17	60.13 ± 1.66	19.43 ± 0.56	32.38 ± 0.49	196.00 ± 16.92	2.95 ± 0.46
p-value		0.824	0.830	0.671	0.028	0.002	0.002	0.838	0.535	0.745
Total	n=20	7.04 ± 0.27	21.63 ± 0.71	8.27 ± 1.01	3.86 ± 0.16	56.65 ± 1.48	18.34 ± 0.47	32.45 ± 0.35	205.003 ± 17.14	2.87 ± 0.29
β-thalassemia/Hb E	n=30	7.07 ± 0.27	22.26 ± 0.72	30.44 ± 8.60	3.73 ± 0.15	60.62 ± 1.59	19.11 ± 0.47	31.66 ± 0.38	356.43 ± 46.23	6.03 ± 1.41

*Values were given as mean ± SEM

Table 9 Hematological data of normal subjects compared with β-thalassemia/Hb E patients

Subjects	Hematological data*								
	Hb g/dl	**Hct** %	**WBC** 10^3/ml	**RBC** 10^6/ml	**MCV** fl	**MCH** pg	**MCHC** g/dl	**platelet** 10^3/ml	**reticulocyte** %
Normal control (N=30)	14.26 ± 0.29	13.81 ± 0.22	6.23 ± 0.30	4.82 ± 0.85	88.40 ± 0.47	28.63 ± 0.90	33.42 ± 0.17	248.97 ± 8.06	1.14 ± 0.07
β-thalassemia/Hb E (N=30)	7.07 ± 0.27	22.26 ± 0.72	30.44 ± 8.60	3.73 ± 0.15	60.62 ± 1.59	19.11 ± 0.47	31.66 ± 0.38	356.43 ± 46.23	6.03 ± 1.41
p-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	0.535	<0.001
Splenectomized (N=10)	7.13 ± 0.62	23.52 ± 1.61	74.78 ± 19.56	3.48 ± 0.31	68.57 ± 2.17	20.65 ± 0.90	30.09 ± 0.69	659.30 ± 64.27	13.06 ± 3.63
Non-splenectomized (N=20)	7.04 ± 0.27	21.63 ± 0.71	8.27 ± 1.01	3.86 ± 0.16	56.65 ± 1.48	18.34 ± 0.47	32.45 ± 0.35	205.003 ± 17.14	2.87 ± 0.29
p-value	0.871	0.303	0.008	0.231	<0.001	0.017	0.002	<0.001	0.023

*Values were given as mean ± SEM

2. Glutathione in whole blood by HPLC

The conjugation of glutathione and bromobimane fluorescence produced glutathione-S-bimane. A HPLC with fluorescence detector was used to determine GSH. Chromatogram of HPLC showed a peak of glutathione which was eluted at 3.75 minutes at flow rate of 1.5 ml/min. The internal standard cysteamine 50 μM was eluted at 4.18 minutes (Figure 18) in the same injection.

Glutathione and internal standard in whole blood sample of normal subjects and patients were eluted out the same as in standard injection. The chromatograms of normal subject and β-thalassemia/Hb E patient were shown in Figures 19 and 20, respectively. The total glutathione included reduced and oxidized forms. Reduced glutathione was obtained from a subtraction of total glutathione and oxidized glutathione.

3. Linearity range and limit of detection

A linearity relation was obtained from glutathione standard concentration in the range of 20, 40, 60, 80, 90, 100 μmol/L (Figure 21). The equation for the linear regression was $y=6.06x10^{-2}x$; whereas x is the peak area and y is the concentration of analyte. The lowest concentration of the linearity study was above the limit of detection 50 nmol/L.

4. Accuracy, precision, and recovery studies on HPLC detection

The accuracy or intra-assay of whole blood glutathione was studied at 8 different times. The results were shown in Table 10 and Figure 22. At initial time, total glutathione was 861.00 and GSSG was 147.00 μmol/L. The subtraction between total glutathione and GSSG was GSH (714.00 μmol/L). As time increased, the reduced glutathione was decreased while GSSG was increased. The coefficient variations (CV) for total glutathione and reduced glutathione were 2.56% and 8.09%, respectively.

The precision of study is a degree of reproducibility. A study during 8 different days within 1 month was shown in Table 11. Oxidized glutathione was slightly increased during a day with a concomitant decrease in GSH. The CVs of

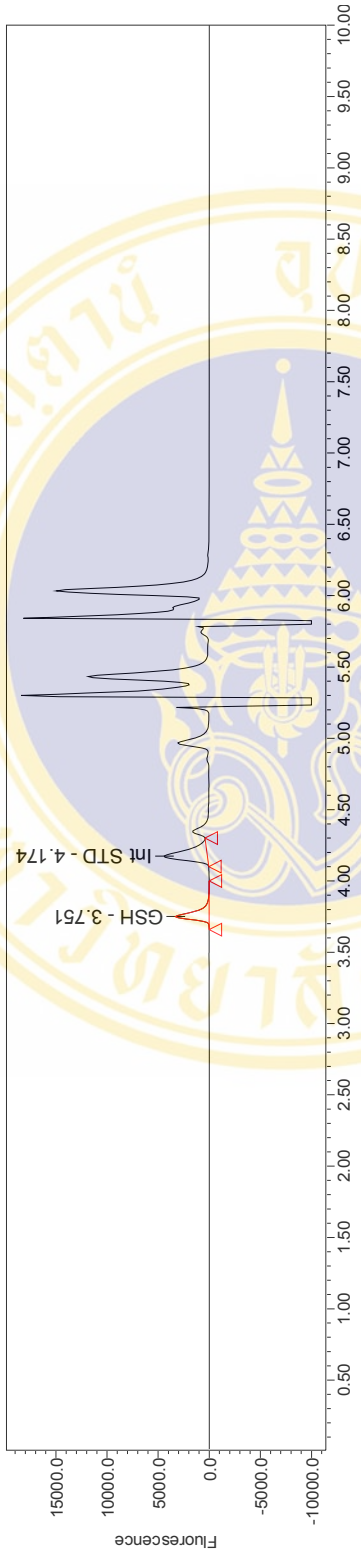


Figure 18 Chromatogram of reduced glutathione standard. Glutathione (40 µM of standard glutathione) was eluted at 3.751 minutes. IntSTD; internal standard (50 µM of cysteamine) was eluted at 4.174 minutes. Peak eluted after 4.5 minutes was nonspecific compounds related to bromobimane fluorescence.

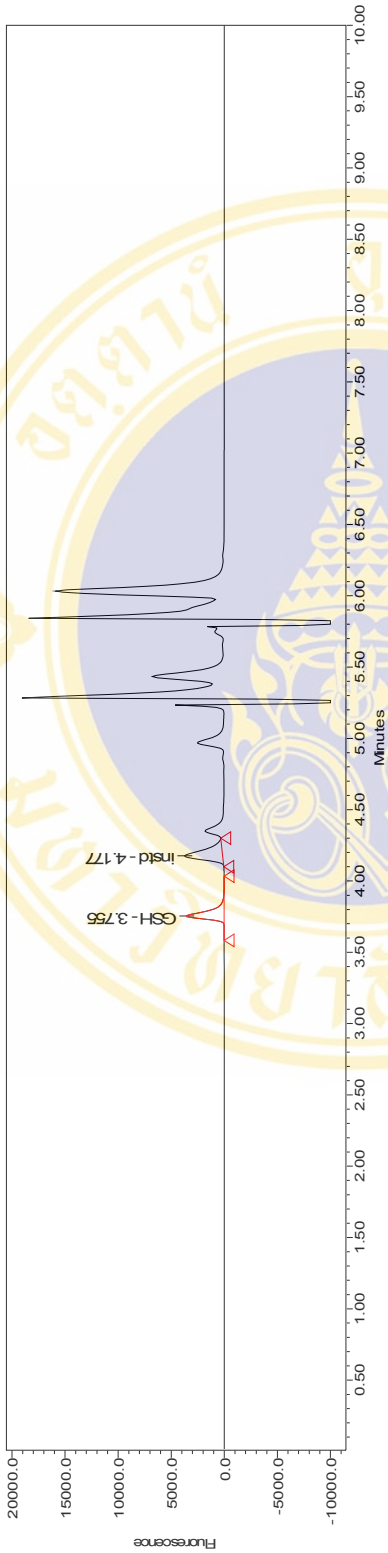


Figure 19 Chromatogram of total glutathione in normal subject. Glutathione was eluted at 3.755 minutes. IntSTD; internal standard (50 µM of cysteamine) was eluted at 4.177 minutes. Peak eluted after 4.5 minutes was nonspecific compounds related to bromobimane fluorescence.

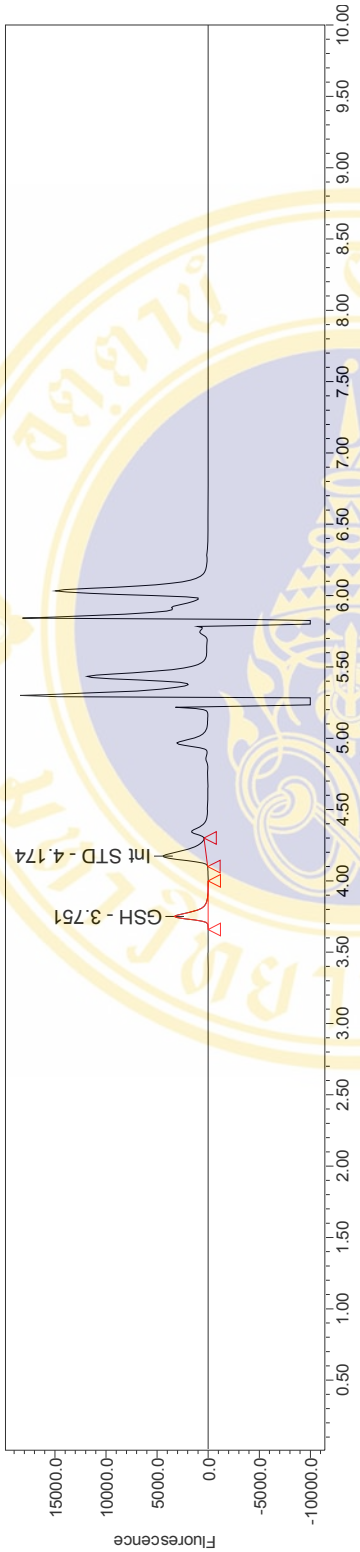


Figure 20 Chromatogram of total glutathione in β-thalassemia/Hb E patient. Glutathione was eluted at 3.751 minutes. IntSTD; internal standard (50 µM of cysteamine) was eluted at 4.174 minutes. Peak eluted after 4.5 minutes was nonspecific compounds related to bromobimane fluorescence

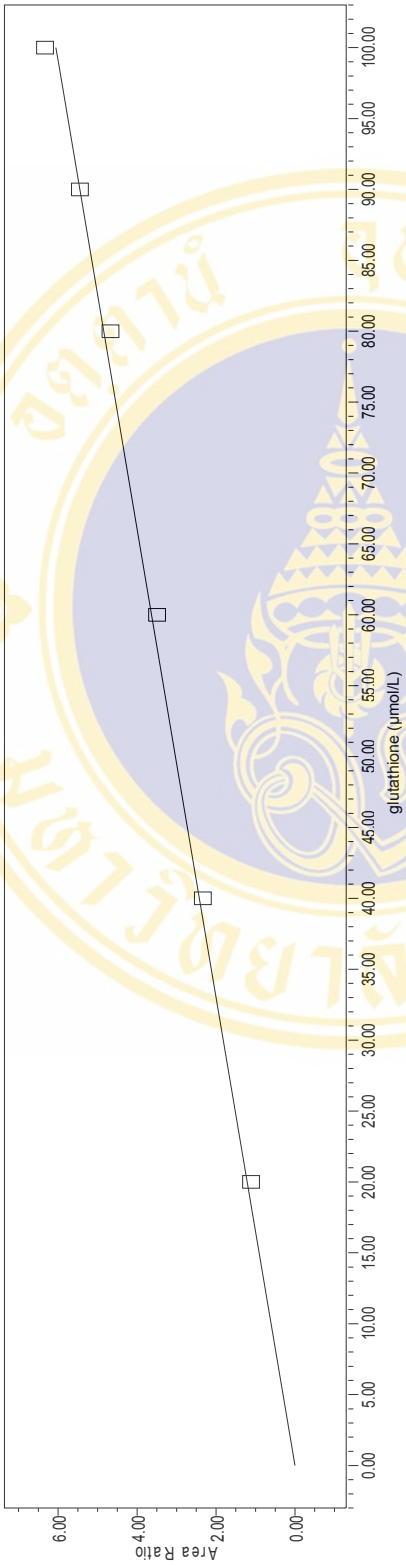


Figure 21 Standard curve of reduced glutathione (20-100 µmol/L)

Table 10 Accuracy or intra-assay of HPLC in whole blood of normal subjects

	Various forms of glutathione		
Minutes	tGSH	GSH	GSSG
0	861.00	714.00	147.00
5	846.50	701.50	145.00
15	815.00	640.50	174.50
30	820.00	643.00	177.00
45	870.50	638.50	232.00
60	835.00	589.00	246.00
120	838.50	602.00	236.50
180	871.50	567.00	304.50
Mean $\pm$ SEM	844.75 $\pm$ 7.65	636.94 $\pm$ 18.21	207.81 $\pm$ 19.78
%RSD	2.56	8.09	26.93

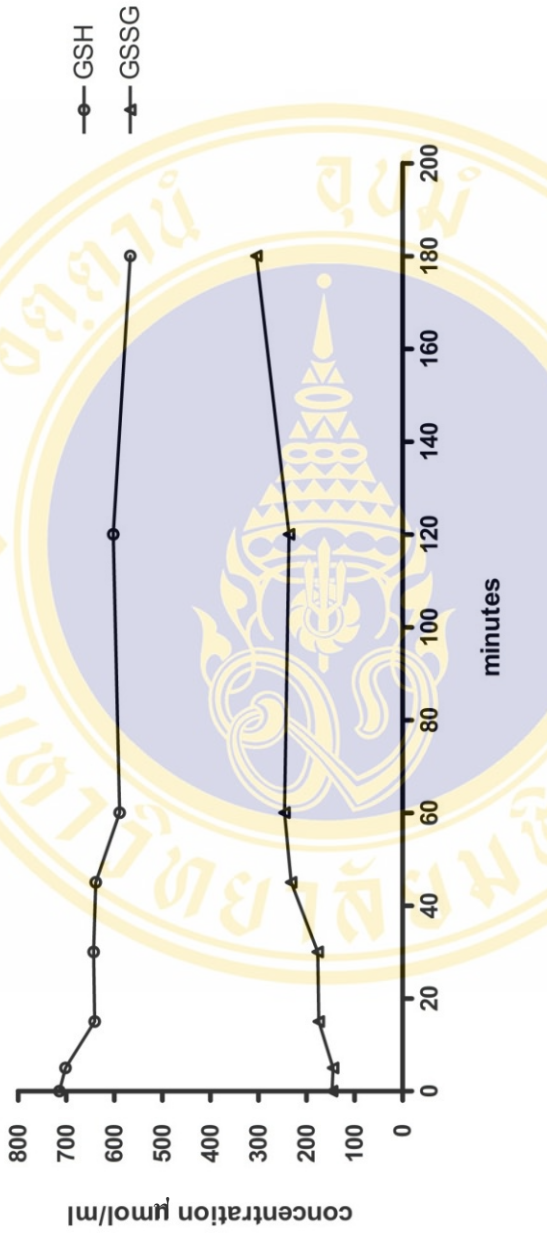


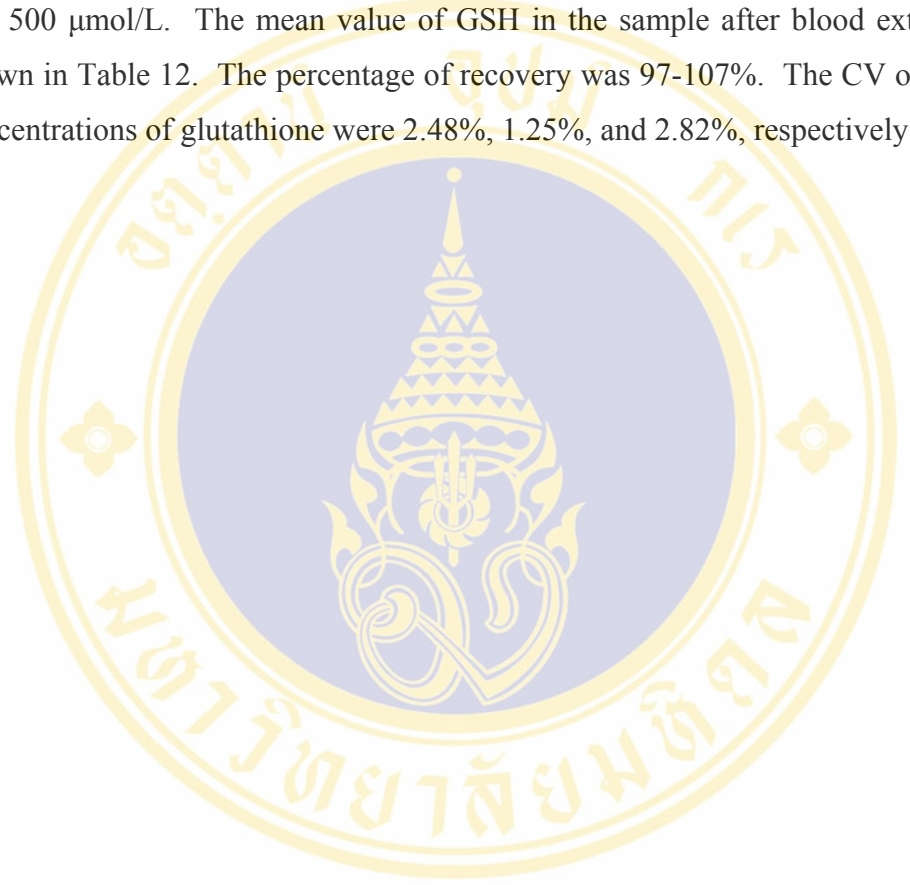
Figure 22 Glutathione (GSH) and oxidized glutathione (GSSG) level during intra-assay determination at 8 different times

Table 11 Precision or inter-assay of HPLC in whole blood of normal subjects

Days	Various forms of glutathione		
	tGSH	GSH	GSSG
0	835.00	686.00	149.00
1	826.50	679.50	147.00
3	823.50	667.50	156.00
4	807.50	659.50	148.00
5	796.00	643.00	153.00
6	795.50	629.50	166.00
7	783.00	618.00	165.00
8	759.00	605.50	153.50
Mean ± SEM	803.25 ± 8.92	648.56 ± 10.37	154.69 ± 2.59
%RSD	3.14	4.52	4.74

inter-assay of total glutathione and reduced glutathione were 3.14% and 4.52%, respectively (Figure 23).

Known concentrations of GSH standard were added into the whole blood samples. The glutathione concentration in the sample was determined in 6 replicates and measured at the same time. The 3 different concentrations added were 150, 250, and 500 μmol/L. The mean value of GSH in the sample after blood extraction was shown in Table 12. The percentage of recovery was 97-107%. The CV of 3 different concentrations of glutathione were 2.48%, 1.25%, and 2.82%, respectively.



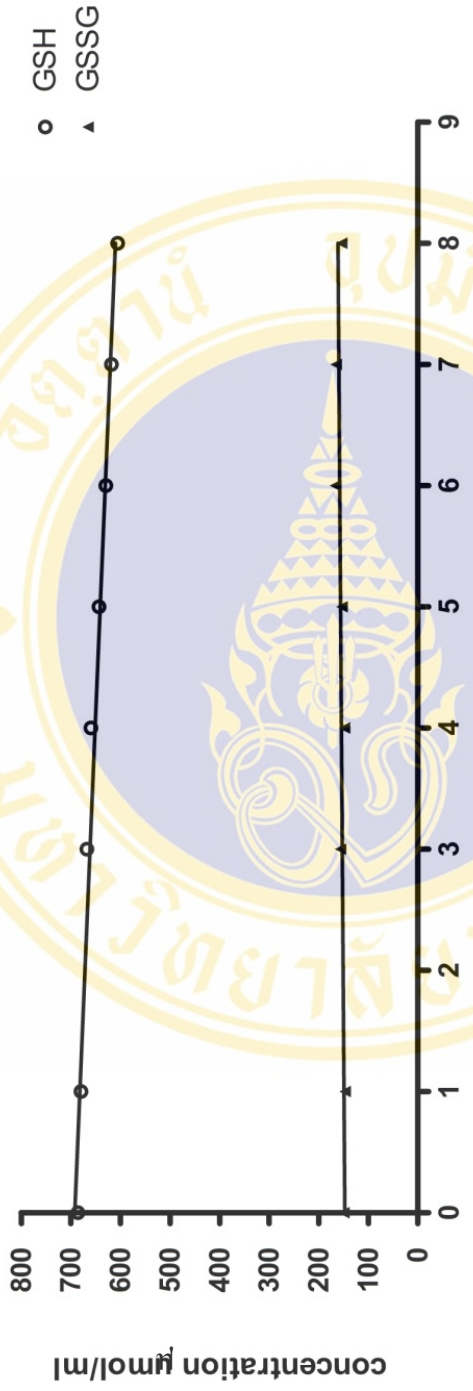


Figure 23 Glutathione (GSH) and oxidized glutathione (GSSG) level during inter-assay determination at 8 different days

Table 12 Recovery rate of known GSH concentration in whole blood samples

Assay	samples	Addition of glutathione concentration (µmol/L)		
		150	250	500
1	599.92	716.11	834.54	1190.90
2	599.65	754.12	841.19	1254.64
3	606.11	770.67	842.98	1234.73
4	603.62	736.11	840.83	1230.34
5	601.67	752.23	814.89	1185.79
6	613.69	746.00	835.29	1273.84
n	6	6	6	6
Mean ± SEM	604.11 ± 2.16	745.87 ± 7.54	834.96 ± 4.25	1228.37 ± 14.17
%RSD		2.48	1.25	2.82
Mean %recovery	-	98.91	97.76	107.86

5. Glutathione concentration, redox ratio and antioxidant enzyme activity in normal subjects

Concentration of glutathione (total glutathione, GSH, and GSSG) were determined in whole blood whereas antioxidant enzyme (GST, GR, and GPX) activity was analyzed in red blood cell. The value of glutathione was expressed as mean ± SEM. The activity of enzyme: GST and GR were expressed as unit per mg Hb, and GPX expressed as unit per g of Hb (Table 13). An average of antioxidant enzyme activity in red blood cell of normal subjects; GST, GR and GPX were 5.37 ± 0.43, 4.61 ± 0.22 U/mg Hb and 31.05 ± 1.39 U/g Hb, respectively. The mean concentration value of total glutathione, GSH, and GSSG were 838.22 ± 27.59, 574.33 ± 20.97, and 263.89 ± 17.24 μmol/L, respectively (Table 14). The concentration of GSH and GSSG were used to define as a redox ratio. A redox ratio of normal subjects was about 2.48 ± 0.02. The mean concentration of GSH in males were 620.22 ± 29.13 μmol/L which was higher than females 537.04 ± 27.00 μmol/L (p=0.046).

6. Glutathione concentration, redox ratio and antioxidant enzymes activity in β-thalassemia/Hb E patients

The data of patients were shown in Tables 15 and 16, all data were expressed as mean ± SEM. The GR activity in males of SP β-thalassemia/Hb E patients was significant lower than in females (p=0.034) (Table 15), whereas other parameters were not significantly different between males and females in both groups of patients (Table 17). The hemoglobin levels in SP and NS β-thalassemia/Hb E patients were not significantly different (7.24 ± 0.51 and 7.04 ± 0.27 g/dl, respectively). The concentrations of total glutathione, GSH, and GSSG in SP patients were 520.33 ± 58.27, 221.98 ± 52.06 and 298.35 ± 25.39 μmol/L, respectively and in the NS patients, the levels were 662.21 ± 45.43, 318.49 ± 28.71 and 343.72 ± 23.90 μmol/L, respectively (Table 17). The redox ratio was 0.79 ± 0.20 in SP patients and 0.96 ± 0.17 in NS patients. The enzymatic antioxidants in red blood cell of SP patients were also not significant difference in NS patients (Table 14). The activity of enzyme GST, GR and GPX were 10.63 ± 1.28, 9.26 ± 1.26 U/mg Hb and 59.68 ± 3.88 U/g Hb in SP and 13.62 ± 0.84, 7.31 ± 0.69 U/mg Hb and 60.13 ± 2.37 U/g Hb in NS patients (p=0.054, 0.152, 0.917, respectively).

Table 13 Concentration of glutathione, redox ratio (GSH/GSSG), enzymes activity (GST, GR, GPX) of normal subjects

Subjects	Sex	Age	Glutathione and antioxidant enzyme status							
			Hb	Total GSH	GSH	GSSG	GSH/GSSG	GST	GR	GPX
			g/dl	µmol/L	µmol/L	µmol/L	Redox ratio	U/mg Hb	U/mg Hb	U/g Hb
CHT	M	24	17.00	754.35	476.35	278.00	1.71	5.70	6.49	31.01
SAI	M	38	16.10	700.50	543.50	157.00	3.46	3.79	4.02	29.46
NAT	M	20	15.40	767.00	568.00	199.00	2.85	8.28	4.49	24.56
PAI	M	24	15.50	814.50	634.50	180.00	3.53	1.72	3.99	34.03
PIS	M	24	16.30	905.00	609.00	296.00	2.06	11.60	6.24	39.46
KRT	M	24	15.40	899.50	673.00	226.50	2.97	3.81	5.01	42.71
ADS	M	26	15.50	815.00	649.50	165.50	3.92	5.49	2.02	27.86
CHP	M	49	13.60	783.00	623.50	159.50	3.91	7.51	4.38	34.79
PRH	M	22	15.90	732.50	494.00	238.50	2.07	3.04	3.84	27.11
AEP	M	23	16.10	1041.00	587.00	454.00	1.29	1.83	3.84	19.04
TNS	M	24	15.90	1022.50	609.00	413.50	1.47	6.92	3.58	39.59
KOM	M	24	13.30	932.50	700.50	232.00	3.02	4.86	4.68	29.74
SRV	M	24	15.80	1365.00	895.00	470.00	1.90	6.77	5.26	29.46
WTC	M	24	14.30	1869.00	1401.00	468.00	2.99	2.85	4.13	46.38
Mean ± SEM	(n=14)		15.44 ± 0.28	887.10 ± 49.68	620.22 ± 29.13	266.88 ± 30.85	2.63 ± 0.26	5.30 ± 0.74	4.43 ± 0.30	32.51 ± 2.00

Table 13 Concentration of glutathione, redox ratio (GSH/GSSG), enzymes activity (GST, GR, GPX) of normal subjects (continued)

Subjects	Sex	Age	Glutathione and antioxidant enzyme status							
			Hb	Total GSH	GSH	GSSG	GSH/GSSG	GST	GR	GPX
			g/dl	µmol/L	µmol/L	µmol/L	Redox ratio	U/mg Hb	U/mg Hb	U/g Hb
RTY	F	40	13.90	897.45	767.95	129.50	5.93	7.71	4.66	21.54
SUP	F	29	11.50	920.95	615.95	305.00	2.02	4.41	5.90	36.73
PCG	F	24	12.40	1071.00	706.00	365.00	1.93	4.50	6.30	28.14
AUK	F	36	12.10	724.10	467.65	256.45	1.82	4.45	4.45	35.46
PAN	F	24	13.40	745.25	508.15	237.10	2.14	4.84	4.84	31.84
KNT	F	24	12.70	685.25	446.40	238.85	1.87	4.07	4.07	23.21
BSP	F	26	12.50	711.45	492.55	218.90	2.25	7.02	7.02	20.94
WAP	F	50	13.00	782.00	560.25	221.75	2.53	6.55	3.04	26.31
SUG	F	22	15.10	646.00	323.50	322.50	1.00	4.63	4.44	25.96
PRA	F	39	13.60	834.00	436.00	398.00	1.10	3.97	2.86	26.83
ARP	F	24	13.60	822.00	616.70	205.30	3.00	3.98	3.90	43.02
SUK	F	22	15.10	825.50	569.00	256.50	2.22	9.20	6.37	47.06
SUC	F	23	13.80	792.00	567.00	225.00	2.52	7.15	5.18	19.37
PAP	F	24	11.60	853.00	460.50	392.50	1.17	8.50	3.13	27.31
SKN	F	24	15.10	808.00	540.00	268.00	2.01	3.99	4.62	31.22
TNP	F	35	12.20	658.00	515.00	143.00	3.60	1.99	5.63	31.39
Mean ± SEM	(n=16)		13.23 ± 0.30	798.50 ± 27.14	537.04 ± 27.00	261.46 ± 19.65	2.32 ± 0.29	5.44 ± 0.50	4.78 ± 0.31	29.77 ± 1.94

Table 14 Comparison of glutathione concentration, redox ratio (GSH/GSSG), enzymes activity (GST, GR, GPX) of normal subjects between males and females

Subjects		Glutathione and antioxidant enzyme status*							
		Hb	**Total GSH**	**GSH**	**GSSG**	**GSH/GSSG**	**GST**	**GR**	**GPX**
		g/dl	**µmol/L**	**µmol/L**	**µmol/L**	**Redox ratio**	**U/mg Hb**	**U/mg Hb**	**U/g Hb**
Normal subjects									
Male	(n=14)	15.44 ± 0.28	887.10 ± 49.68	620.22 ± 29.13	266.88 ± 30.85	2.63 ± 0.26	5.30 ± 0.74	4.43 ± 0.30	32.51 ± 2.00
Female	(n=16)	13.23 ± 0.30	798.50 ± 27.14	537.04 ± 27.00	261.46 ± 19.65	2.32 ± 0.29	5.44 ± 0.50	4.78 ± 0.31	29.77 ± 1.94
p-value		0.001	0.112	0.046	0.879	0.447	0.876	0.427	0.334
Total	(n=30)	14.26 ± 0.29	838.22 ± 27.59	574.33 ± 20.97	263.89 ± 17.24	2.46 ± 0.20	5.37 ± 0.43	4.61 ± 0.22	31.05 ± 1.39

*Values were given as mean ± SEM

Table 15 Concentration of glutathione, redox ratio (GSH/GSSG), enzymes activity (GST, GR, GPX) of splenectomized β-thalassemia/Hb E patients

Subjects	Sex	Age	Type	Glutathione and antioxidant enzyme status							
				Hb	Total GSH	GSH	GSSG	GSH/GSSG	GST	GR	GPX
				g/dl	µmol/L	µmol/L	µmol/L	Redox ratio	U/mg Hb	U/mg Hb	U/g Hb
NPJ	M	42	SP	9.80	431.00	142.50	288.50	0.49	9.35	5.29	72.22
APR	M	25	SP	7.30	623.50	233.00	390.50	0.60	3.57	2.76	65.65
NPN	M	22	SP	6.50	467.00	190.50	276.50	0.69	12.86	10.25	53.65
PJJ	M	30	SP	5.60	396.00	156.50	239.50	0.65	14.81	6.92	51.91
GRJ	M	20	SP	6.10	359.50	168.00	191.50	0.88	7.26	10.30	51.39
TYT	M	28	SP	8.20	433.50	70.00	363.50	0.19	11.55	7.67	37.49
Mean ± SEM		(n=6)		7.25 ± 0.63	451.75 ± 37.47	160.08 ± 22.17	291.67 ± 30.51	0.58 ± 0.09	9.90 ± 1.66	7.20 ± 1.19	55.39 ± 4.99
VPK	F	21	SP	4.70	417.55	70.95	346.60	0.20	7.41	8.46	60.50
PLP	F	17	SP	7.60	392.50	189.50	203.00	0.93	9.00	13.74	74.69
CTM	F	30	SP	5.00	875.00	618.50	256.50	2.41	13.39	16.41	53.97
APT	F	44	SP	10.50	807.70	380.30	427.40	0.89	17.06	10.79	75.31
Mean ± SEM		(n=4)		6.95 ± 1.35	623.19 ± 126.81	314.81 ± 119.61	308.38 ± 49.52	1.11 ± 0.47	11.72 ± 2.18	12.35 ± 1.73	66.12 ± 5.30
Total		(n=10)		7.13 ± 0.62	520.33 ± 58.27	221.98 ± 52.06	298.35 ± 25.39	0.79 ± 0.20	10.63 ± 1.28	9.26 ± 1.26	59.68 ± 3.88

Table 16 Concentration of glutathione, redox ratio (GSH/GSSG), enzymes activity (GST, GR, GPX) of non-splecnectomized β-thalassemia/Hb E patients

Subjects	Sex	Age	Type	Glutathione and antioxidant enzyme status							
				Hb	Total GSH	GSH	GSSG	GSH/GSSG	GST	GR	GPX
				g/dl	µmol/L	µmol/L	µmol/L	Redox ratio	U/mg Hb	U/mg Hb	U/g Hb
CNB	M	45	NS	8.70	390.50	147.50	243.00	0.61	20.59	8.41	61.99
SRP	M	52	NS	8.50	828.95	429.55	399.40	1.08	11.94	2.94	53.24
VSB	M	16	NS	6.80	786.60	398.70	387.90	1.03	11.44	6.21	55.26
TSC	M	19	NS	6.20	440.00	221.50	218.50	1.01	8.15	8.77	65.01
DNN	M	18	NS	7.50	845.80	404.10	441.70	0.91	11.48	8.73	42.85
SPS	M	41	NS	6.30	787.15	422.90	364.25	1.16	14.42	9.55	43.66
TNK	M	39	NS	6.80	846.25	383.00	463.25	0.83	19.86	10.97	49.11
SYU	M	27	NS	6.10	788.30	446.80	341.50	1.31	11.34	12.82	71.23
Mean ± SEM		(n=8)		7.11 ± 0.36	714.19 ± 66.00	356.76 ± 38.86	357.4 ± 30.96	0.99 ± 0.08	13.65 ± 1.56	8.55 ± 1.05	55.29 ± 3.60

Table 16 Concentration of glutathione, redox ratio (GSH/GSSG), enzymes activity (GST, GR, GPX) of non-splecnectomized β-thalassemia/Hb E patients (continued)

Subjects	Sex	Age	Type	Glutathione and antioxidant enzyme status							
				Hb	Total GSH	GSH	GSSG	GSH/GSSG	GST	GR	GPX
				g/dl	µmol/L	µmol/L	µmol/L	Redox ratio	U/mg Hb	U/mg Hb	U/g Hb
CNP	F	22	NS	6.90	339.00	124.50	214.50	0.58	11.27	4.68	60.87
MNR	F	31	NS	7.90	811.40	597.68	213.72	2.80	19.41	5.87	68.99
YSN	F	54	NS	6.40	446.00	193.00	253.00	0.76	13.89	4.71	74.43
GST	F	30	NS	8.40	822.85	381.85	441.00	0.87	12.52	5.91	75.45
YPN	F	21	NS	6.80	666.50	273.50	393.00	0.70	12.24	8.14	61.43
JTJ	F	35	NS	6.00	359.50	168.00	191.50	0.88	15.48	8.38	69.28
BSP	F	49	NS	5.90	846.10	381.25	464.85	0.82	9.14	13.21	45.40
PGS	F	24	NS	9.10	718.50	255.00	463.50	0.55	8.00	3.01	59.28
APP	F	24	NS	9.40	862.85	362.05	500.80	0.72	19.01	3.49	76.28
PTN	F	28	NS	5.30	440.00	221.50	218.50	1.01	12.43	10.37	57.27
VTY	F	27	NS	6.00	390.50	147.50	243.00	0.61	15.96	6.03	62.74
VPJ	F	34	NS	5.70	827.50	409.96	417.55	0.98	14.12	4.09	48.88
Mean ± SEM		(n=12)		6.98 ± 0.40	627.56 ± 61.81	292.98 ± 39.80	334.58 ± 34.91	0.94 ± 0.17	13.62 ± 1.01	6.49 ± 0.87	63.36 ± 2.88
Total mean		(n=20)		7.04 ± 0.27	662.21 ± 45.43	318.49 ± 28.71	343.72 ± 23.90	0.96 ± 0.11	13.63 ± 0.84	7.31 ± 0.69	60.13 ± 2.37

Table 17 Comparison of glutathione concentartion, redox ratio (GSH/GSSG), enzymes activity (GST, GR, GPX) of β-thalassemia/Hb E patients between males and females

Subjects		Glutathione and antioxidant enzyme status*							
		Hb	Total GSH	GSH	GSSG	GSH/GSSG	GST	GR	GPX
		g/dl	µmol/L	µmol/L	µmol/L	Redox ratio	U/mg Hb	U/mg Hb	U/g Hb
β-thalassemia/Hb E Splenectomized									
Male	(n=6)	7.25 ± 0.63	451.75 ± 37.47	160.08 ± 22.17	291.67 ± 30.51	0.58 ± 0.09	9.90 ± 1.66	7.20 ± 1.19	55.39 ± 4.99
Female	(n=4)	6.95 ± 2.70	623.19 ± 126.81	314.81 ± 119.61	308.38 ± 49.52	1.11 ± 0.47	11.72 ± 2.18	12.35 ± 1.73	66.12 ± 5.30
p-value		0.827	0.273	0.288	0.768	0.212	0.521	0.034	0.190
Total	(n=10)	7.13 ± 0.62	520.33 ± 58.27	221.98 ± 52.06	298.35 ± 25.39	0.79 ± 0.20	10.63 ± 1.28	9.26 ± 1.26	59.68 ± 3.88
β-thalassemia/Hb E Non-splenectomized									
Male	(n=8)	7.11 ± 0.36	714.19 ± 66.00	356.76 ± 38.86	357.4 ± 30.96	0.99 ± 0.08	13.65 ± 1.56	8.55 ± 1.05	55.29 ± 3.60
Female	(n=12)	6.98 ± 0.40	627.56 ± 61.81	292.98 ± 39.80	334.58 ± 34.91	0.94 ± 0.17	13.62 ± 1.01	6.49 ± 0.87	63.36 ± 2.88
p-value		0.824	0.364	0.288	0.630	0.817	0.987	0.151	0.096
Total	(n=20)	7.03 ± 0.27	662.21 ± 45.43	318.49 ± 28.71	343.72 ± 23.90	0.96 ± 0.11	13.63 ± 0.84	7.31 ± 0.69	60.13 ± 2.37
β-thalassemia/Hb E (N=30)		7.07 ± 0.27	614.92 ± 37.51	286.32 ± 26.68	328.59 ± 18.21	0.91 ± 0.10	12.63 ± 0.74	7.96 ± 0.64	59.98 ± 2.00

*Values were given as mean ± SEM

7 Comparison of glutathione concentration, redox ratio and antioxidant enzyme activity between normal subjects and β-thalassemia/Hb E patients

Data of normal subjects and β-thalassemia/Hb E patients were compared in Table 18 and shown as mean ± SEM. All data in normal subjects were statistically significant difference from patients. The mean of total and reduced glutathione in β-thalasemia/Hb E patients was 614.92 ± 37.51 and 286.32 ± 26.68 µmol/L which were significantly lower than in normal subjects 838.22 ± 27.59 and 574.33 ± 20.97 µmol/L (Figures 24 and 25) (p=0.001, and <0.001). During oxidative stress protection, GSH was converted to GSSG, therefore, GSSG in patients increased. The statistically significant difference was observed in GSSG level between normal and patients (Table 18, Figure 26). The level of GSSG in patients was 328.60 ± 18.21 µmol/L which was higher than in normal subjects 263.89 ± 17.24 µmol/L (p=0.024). There was not statistically significance different was found in GSSG between normal and SP patients (p=0.228). A low amount of GSH and high level of GSSG in patients are major causes of decreasing redox ratio. The redox ratio in patients was 2.7-fold lower than in normal subjects (p<0.001).

The correlations between glutathione and hemoglobin level were analyzed in both groups of β-thalasemia/Hb E patients (Figures 27-29). Total glutathione, GSH and GSSG did not have correlation with hemoglobin level in both of SP and NS patients with p>0.05. Redox ratio did not correlate with hemoglobin level in SP and NS patients with R^2=0.070 (p=0.460), R^2=0.004 (p=0.939), respectively (Figure 30).

To protect cell from oxidative injury, all antioxidant enzymes were increased in patients. Antioxidant enzyme activity in β-thalassemia/Hb E patients was significantly higher than in normal subjects. Activity of enzyme was monitored in red blood cell of patients and the activity of GST, GR and GSSG were 12.63 ± 0.74 U/g Hb, 7.96 ± 0.64 U/g Hb and 59.98 ± 2.00 U/mg Hb, respectively, whereas the activity of enzyme GST, GR and GPX in normal subjects were 5.37 ± 0.43 U/g Hb, 4.61 ± 0.22 U/g Hb and 31.05 ± 1.39 U/mg Hb, respectively. The activity of antioxidant enzymes in both groups of patients were shown in Figures 31-33.

There was no correlation between hemoglobin and antioxidant enzyme activity in both group of patients. Red blood cell antioxidant enzyme GST, GR and GPX in

Table 18 Comparison of glutathione concentration, redox ratio (GSH/GSSG), enzymes activity (GST, GR, GPX) between normal subjects and β-thalassemia/Hb E patients

Subjects	Glutathione and antioxidant enzyme status							
	Hb	Total GSH	GSH	GSSG	GSH/GSSG	GST	GR	GPX
	g/dl	µmol/L	µmol/L	µmol/L	Redox ratio	U/mg Hb	U/mg Hb	U/g Hb
Normal subjects (n=30)	14.26 ± 0.29	838.22 ± 27.59	574.33 ± 20.97	263.89 ± 17.24	2.46 ± 0.20	5.37 ± 0.43	4.61 ± 0.22	31.05 ± 1.39
β-thalassemia/Hb E (n=30)	7.07 ± 0.27	614.92 ± 37.51	286.32 ± 26.68	328.59 ± 18.21	0.91 ± 0.10	12.63 ± 0.74	7.96 ± 0.64	59.98 ± 2.00
p-value	<0.001	0.001	<0.001	0.024	<0.001	<0.001	<0.001	<0.001
Splenectomized (n=10)	7.13 ± 0.62	520.33 ± 58.27	221.98 ± 52.06	298.35 ± 25.39	0.79 ± 0.20	10.63 ± 1.28	9.26 ± 1.26	59.68 ± 3.88
Non-splenectomized (n=20)	7.03 ± 0.27	662.21 ± 45.43	318.49 ± 28.71	343.72 ± 23.90	0.96 ± 0.11	13.63 ± 0.84	7.31 ± 0.69	60.13 ± 2.37
p-value	0.871	0.074	0.088	0.247	0.420	0.054	0.152	0.917

*Values were given as mean ± SEM

Table 19 Comparison of glutathione concentration, redox ratio (GSH/GSSG), enzymes activity (GST, GR, GPX) between normal subjects, splenectomized and non-splenectomized β-thalassemia/Hb E patients

Subjects	Glutathione and antioxidant enzyme status							
	Hb	**Total GSH**	**GSH**	**GSSG**	**GSH/GSSG**	**GST**	**GR**	**GPX**
	g/dl	**µmol/L**	**µmol/L**	**µmol/L**	**Redox ratio**	**U/mg Hb**	**U/mg Hb**	**U/g Hb**
Normal subjects (n=30)	14.26 ± 0.29	838.22 ± 27.59	574.33 ± 20.97	263.89 ± 17.24	2.46 ± 0.20	5.37 ± 0.43	4.61 ± 0.22	31.05 ± 1.39
Splenectomized (n=10)	7.13 ± 0.62	520.33 ± 58.27	221.98 ± 52.06	298.35 ± 25.39	0.79 ± 0.20	10.63 ± 1.28	9.26 ± 1.26	59.68 ± 3.88
p-value	<0.001	<0.001	<0.001	0.228	<0.001	0.001	<0.001	<0.001
Normal subjects (n=30)	14.26 ± 0.29	838.22 ± 27.59	574.33 ± 20.97	263.89 ± 17.24	2.46 ± 0.20	5.37 ± 0.43	4.61 ± 0.22	31.05 ± 1.39
Non-splenectomized (n=20)	7.03 ± 0.27	662.21 ± 45.43	318.49 ± 28.71	343.72 ± 23.90	0.96 ± 0.11	13.63 ± 0.84	7.31 ± 0.69	60.13 ± 2.37
p-value	<0.001	0.036	<0.001	0.024	<0.001	<0.001	0.002	<0.001

*Values were given as mean ± SEM

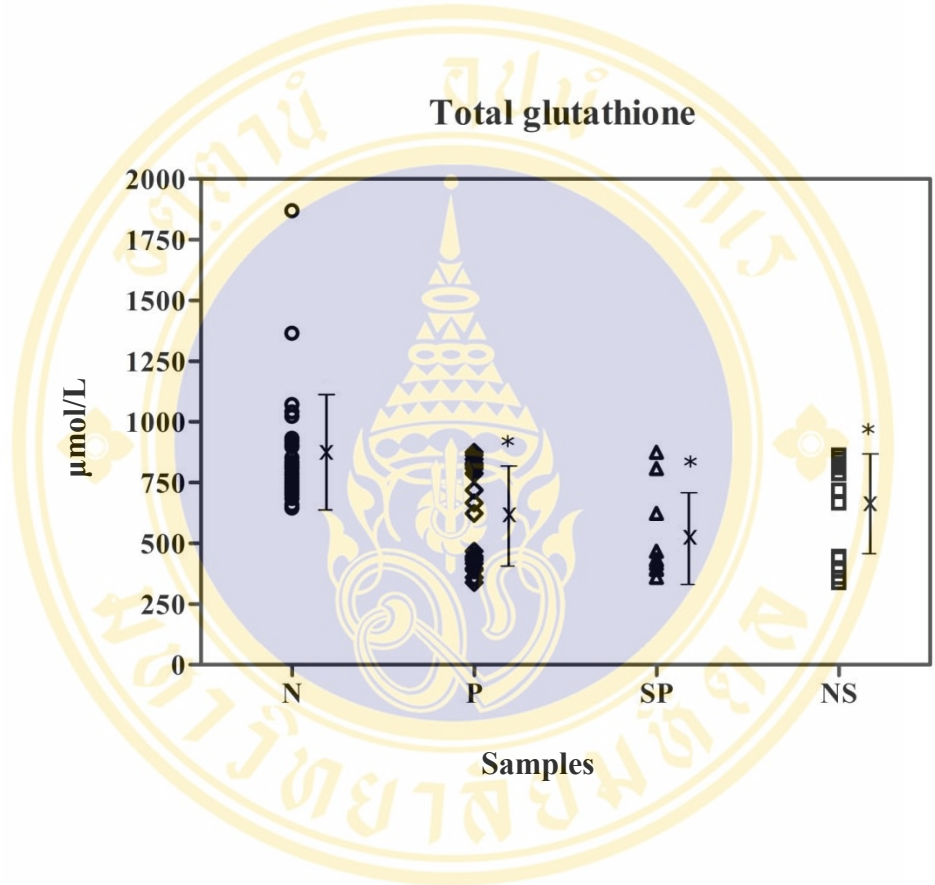


Figure 24 Scattergram of the total glutathione level in whole blood sample of normal subjects (o), β-thalassemia/Hb E (n=30) (◊), splenectomized β-thalassemia/Hb E (n=10) (Δ), and non-splenectomized β-thalassemia/Hb E (n=20) (□). This result is shown as the mean ± SD. * Significantly different from normal subjects with $p<0.05$.

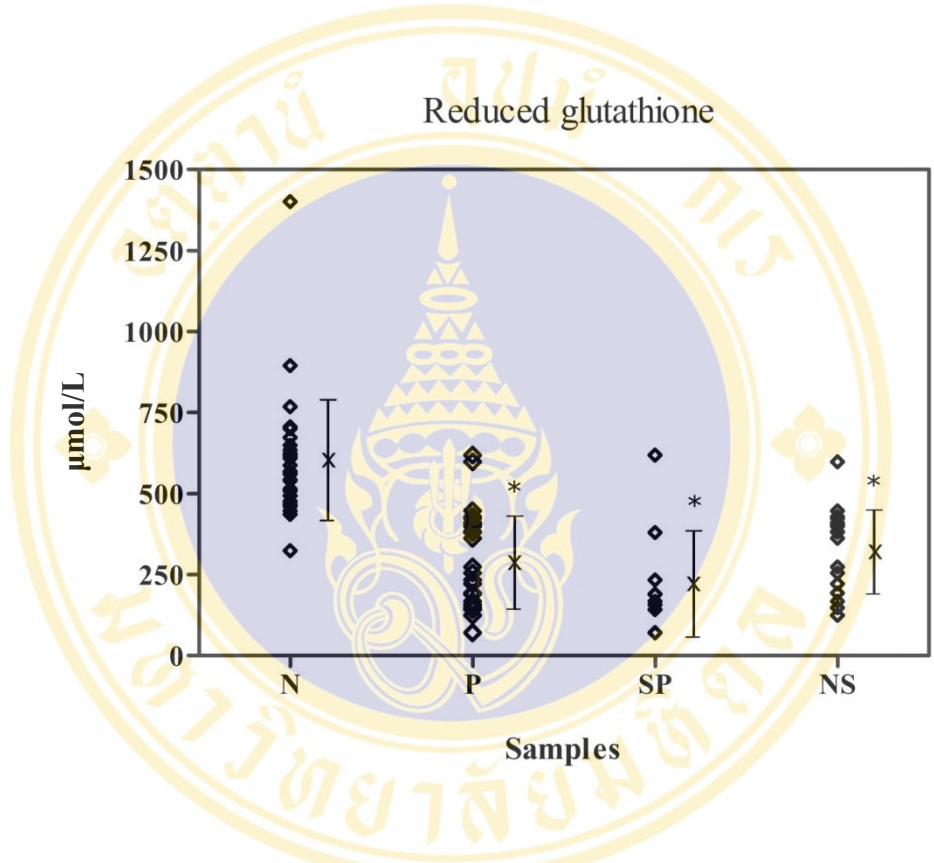


Figure 25 Scattergram of the reduced glutathione level in whole blood sample of normal subjects (o), β-thalassemia/Hb E (n=30) (◊), splenectomized β-thalassemia/Hb E (n=10) (Δ), and non-splenectomized β-thalassemia/Hb E (n=20) (□). This result is shown as the mean ± SD. * Significantly different from normal subjects with $p<0.05$.

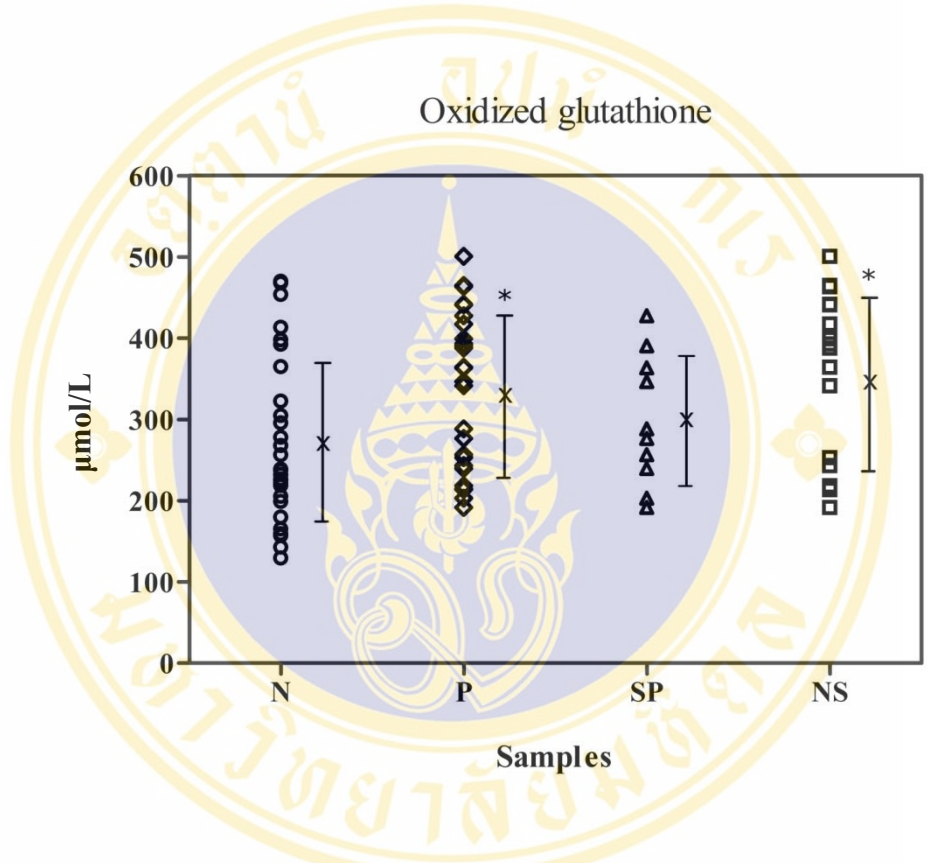


Figure 26 Scattergram of the oxidized glutathione level in whole blood sample of normal subjects (o), β-thalassemia/Hb E (n=30) (◊), splenectomized β-thalassemia/Hb E (n=10) (Δ), and non-splenectomized β-thalassemia/Hb E (n=20) (□). This result is shown as the mean ± SD. * Significantly different from normal subjects with $p<0.05$.

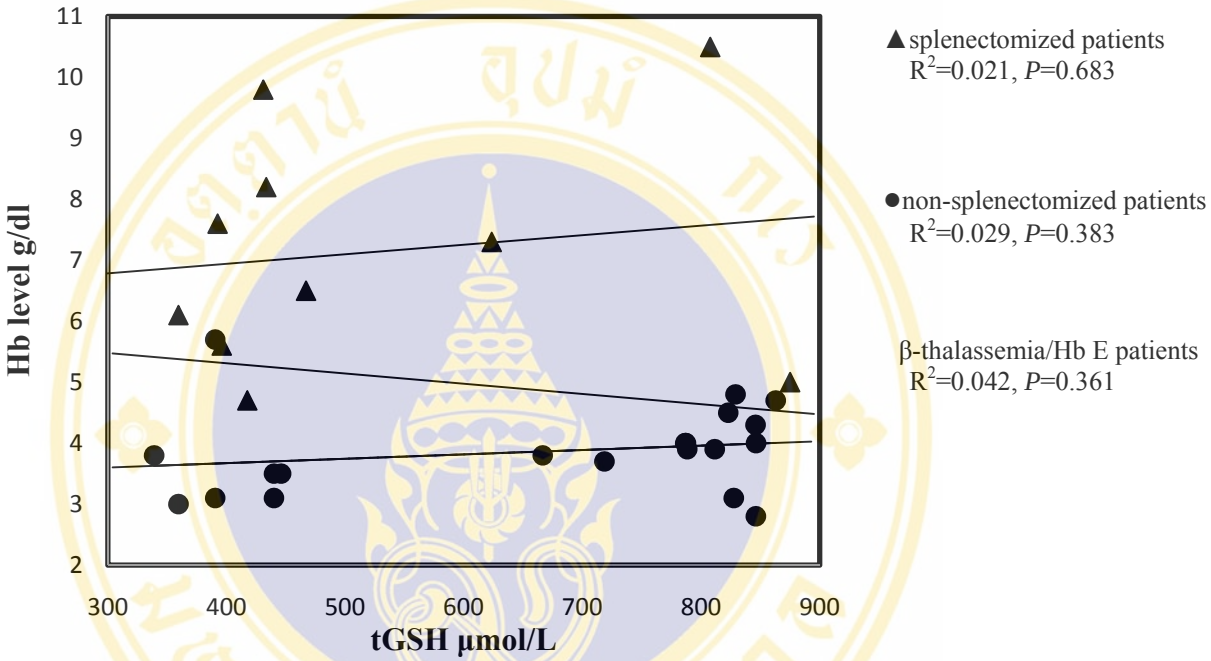


Figure 27 Correlation between hemoglobin (Hb) level and total glutathione (tGSH) concentration in β-thalassemia/Hb E patients (▲ = splenectomized patients, and ● = non-splenectomized patients).

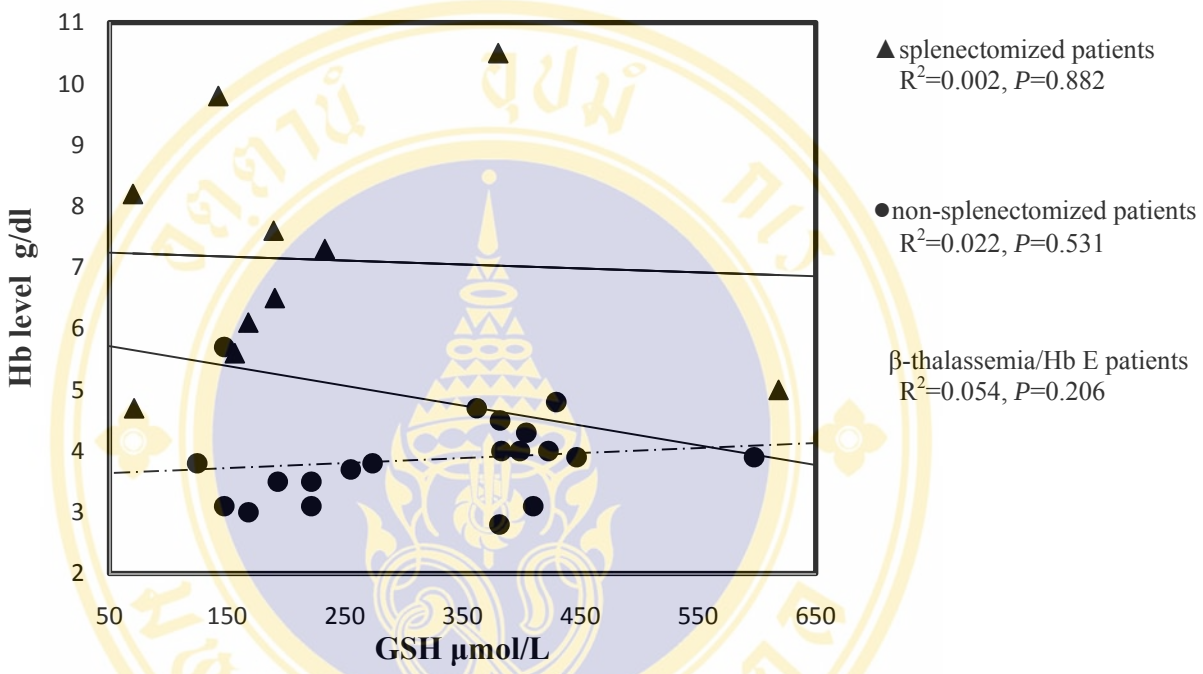


Figure 28 Correlation between hemoglobin (Hb) level and reduced glutathione (GSH) concentration in β-thalassemia/Hb E patients (▲ = splenectomized patients, and ● = non-splenectomized patients).

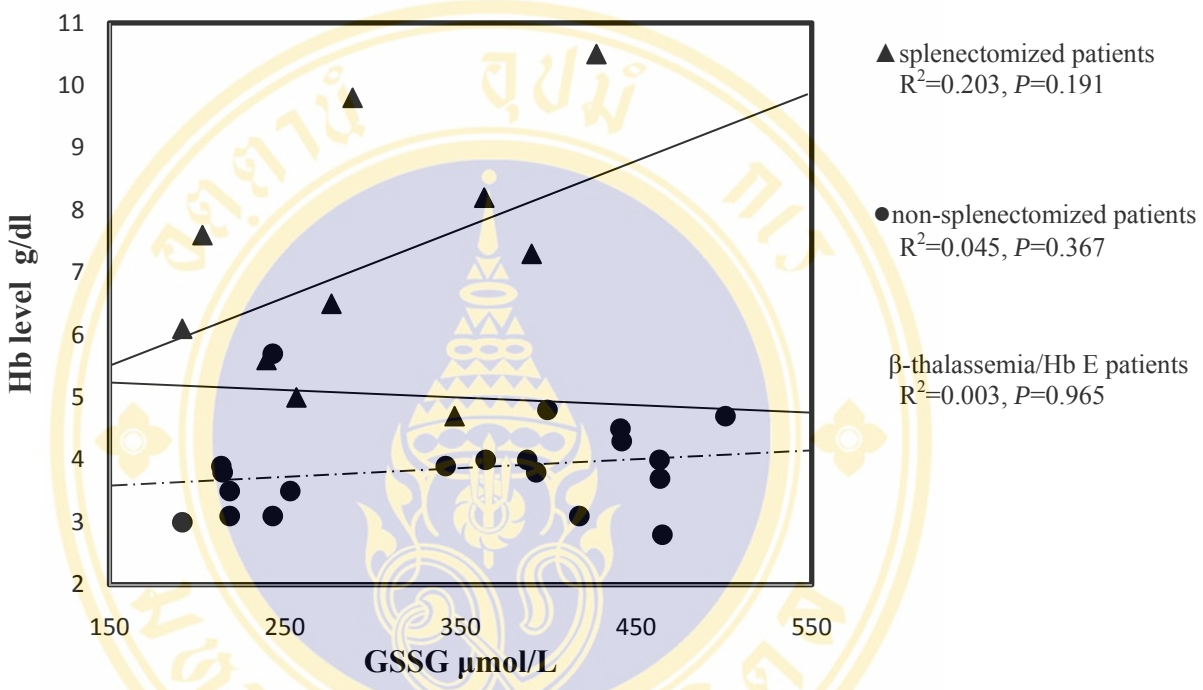


Figure 29 Correlation between hemoglobin (Hb) level and oxidized glutathione (GSSG) concentration in β-thalassemia/Hb E patients (▲ = splenectomized patients, and ● = non-splenectomized patients).

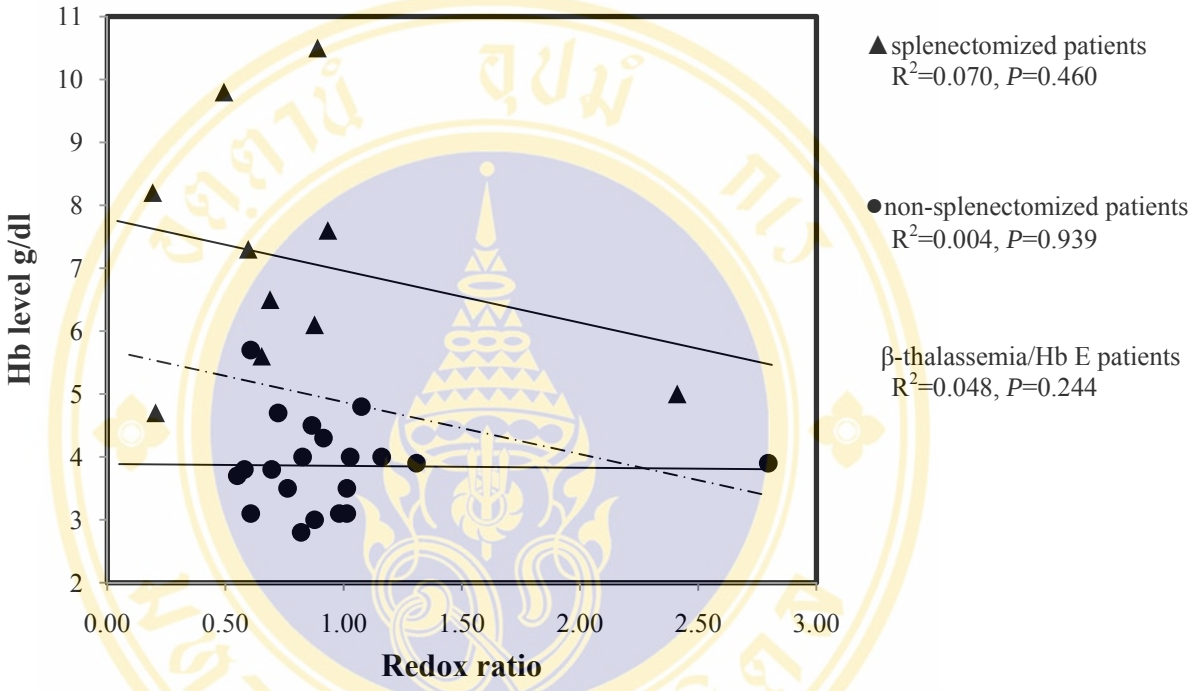


Figure 30 Correlation between hemoglobin (Hb) level and redox ratio (GSH/GSSG) in β-thalassemia/Hb E patients (▲ = splenectomized patients, and ● = non-splenectomized patients).

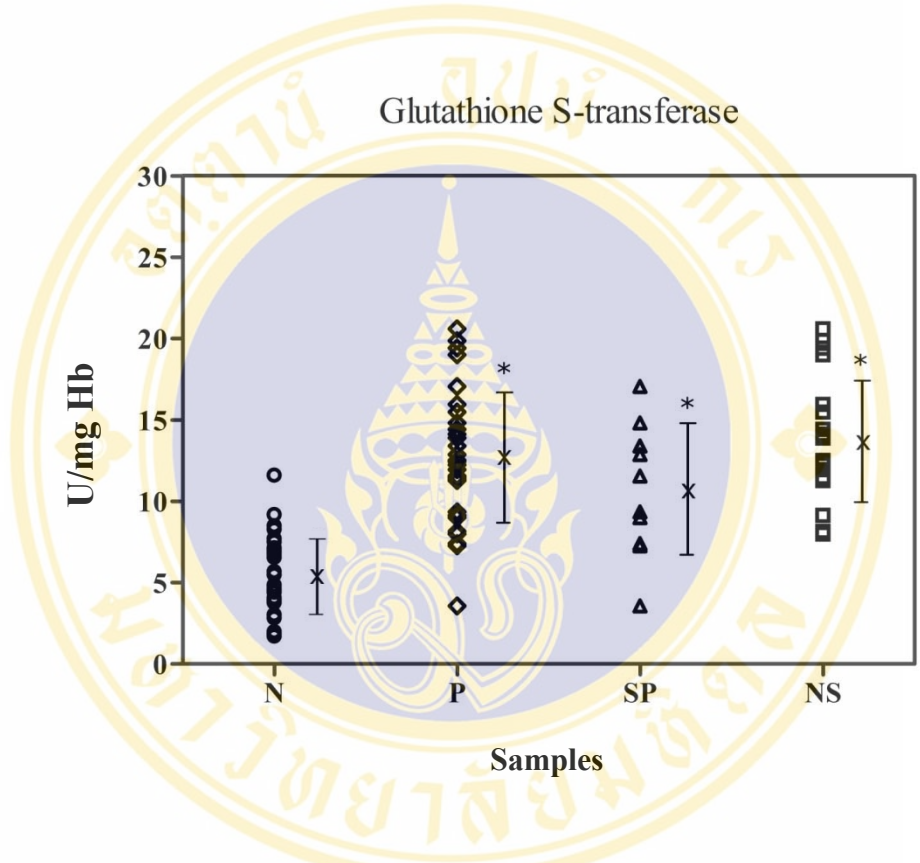


Figure 31 Scattergram of the GST activity in red blood cell sample of normal subjects (**o**), β-thalassemia/Hb E (n=30) (◊), splenectomized β-thalassemia/Hb E (n=10) (Δ), and non-splenectomized β-thalassemia/Hb E (n=20) (□). This result is shown as the mean ± SD. * Significantly different from normal subjects with $p<0.05$.

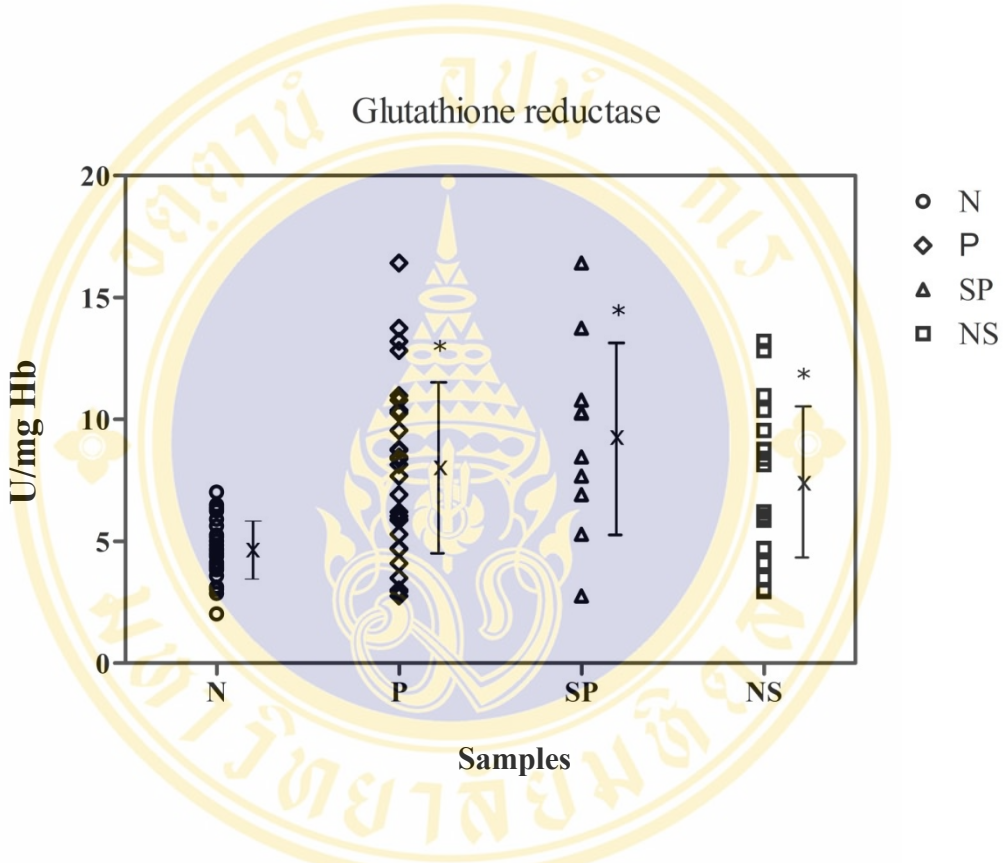


Figure 32 Scattergram of the GR activity in red blood cell sample of normal subjects (**o**), β-thalassemia/Hb E (n=30) (◊), splenectomized β-thalassemia/Hb E (n=10) (Δ), and non-splenectomized β-thalassemia/Hb E (n=20) (□). This result is shown as the mean ± SD. * Significantly different from normal subjects with $p<0.05$.

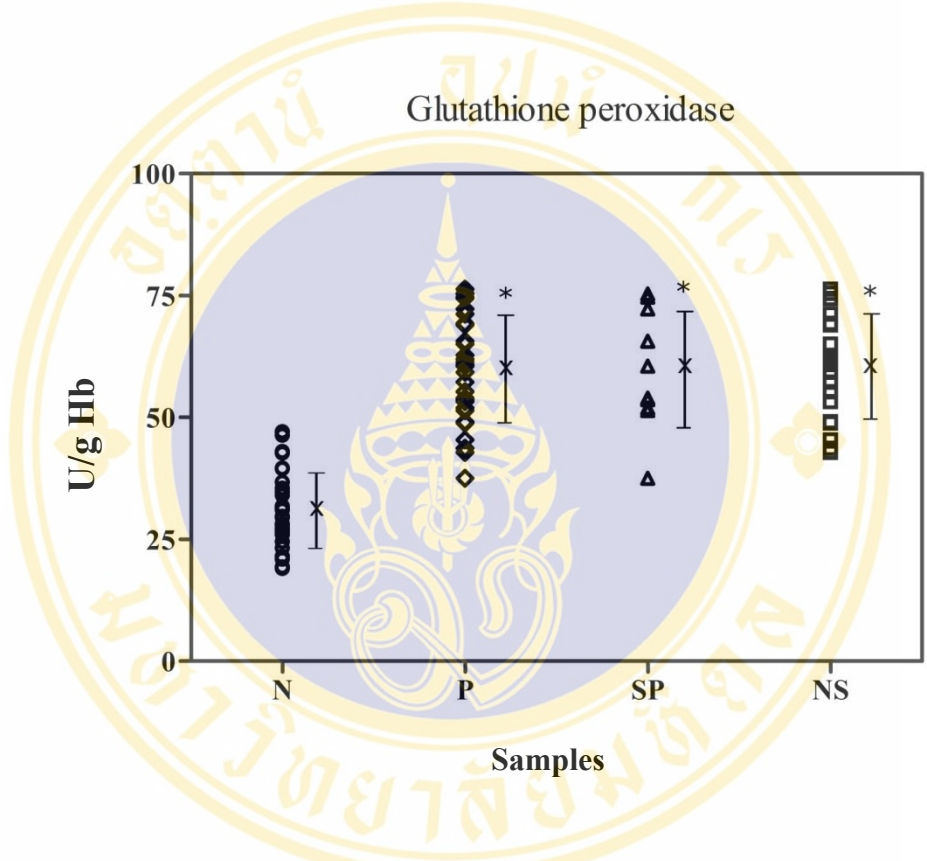


Figure 33 Scattergram of the GPX activity in red blood cell sample of normal subjects (**o),** β-thalassemia/Hb E (n=30) (◊), splenectomized β-thalassemia/Hb E (n=10) (Δ), and non-splenectomized β-thalassemia/Hb E (n=20) (□). This result is shown as the mean ± SD. * Significantly different from normal subjects with $p<0.05$.

SP patients was not correlated with hemoglobin level with R^2=0.043 (p=0.562), R^2=0.063 (p=0.483), R^2=0.238 (p=0.152), respectively. The results were the same as in NS patients, antioxidant enzyme had no correlation with hemoglobin (GST; R^2=0.153, p=0.085, GR; R^2=0.052, p=0.330, GPX; R^2=0.016, p=0.584) (Figures 34-36).

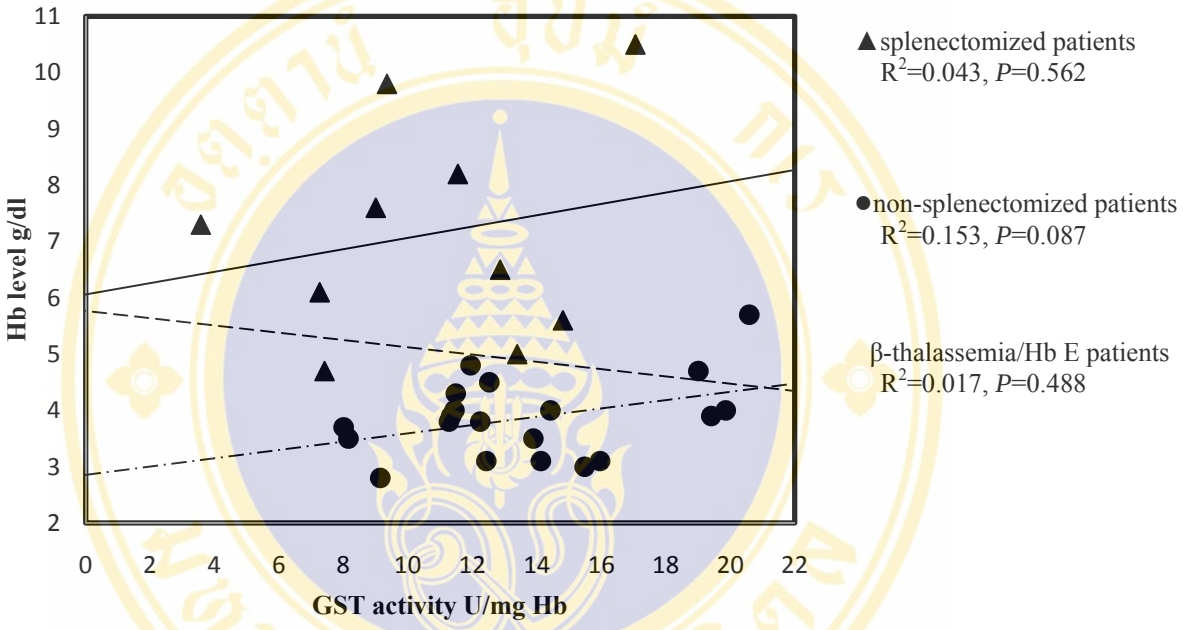


Figure 34 Correlation between hemoglobin (Hb) level and glutathione S-transferase (GST) activity in β-thalassemia/Hb E patients (▲ = splenectomized patients, and ● = non-splenectomized patients).

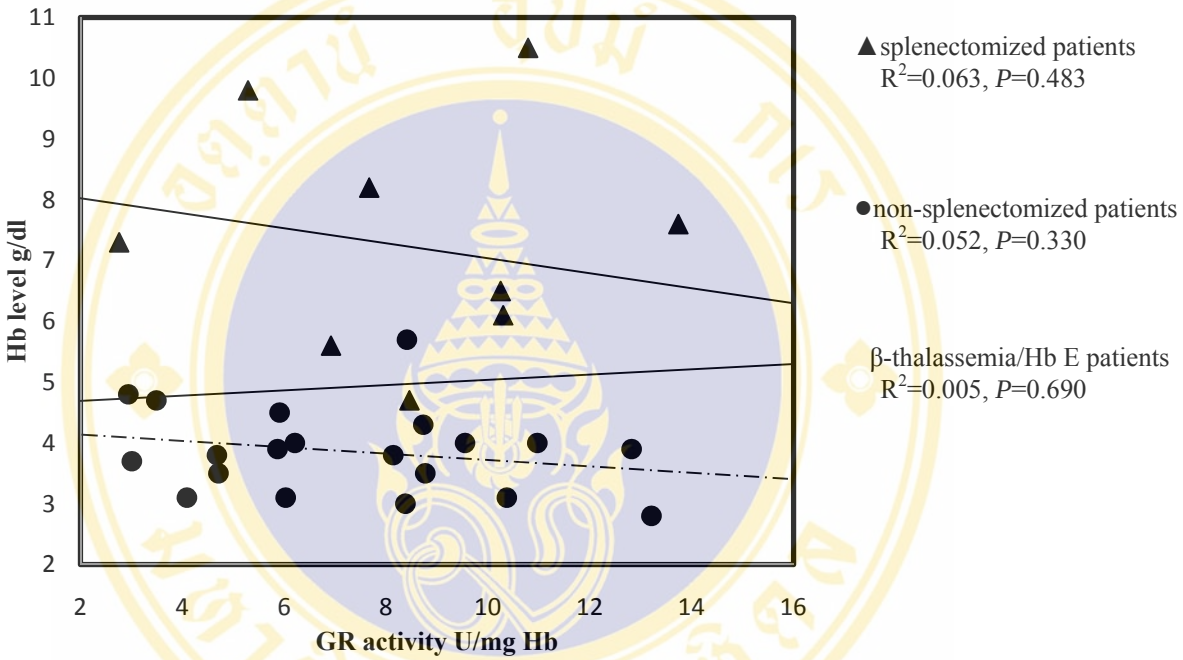


Figure 35 Correlation between hemoglobin (Hb) level and glutathione reductase (GR) activity in β-thalassemia/Hb E patients (▲ = splenectomized patients, and ● = non-splenectomized patients).

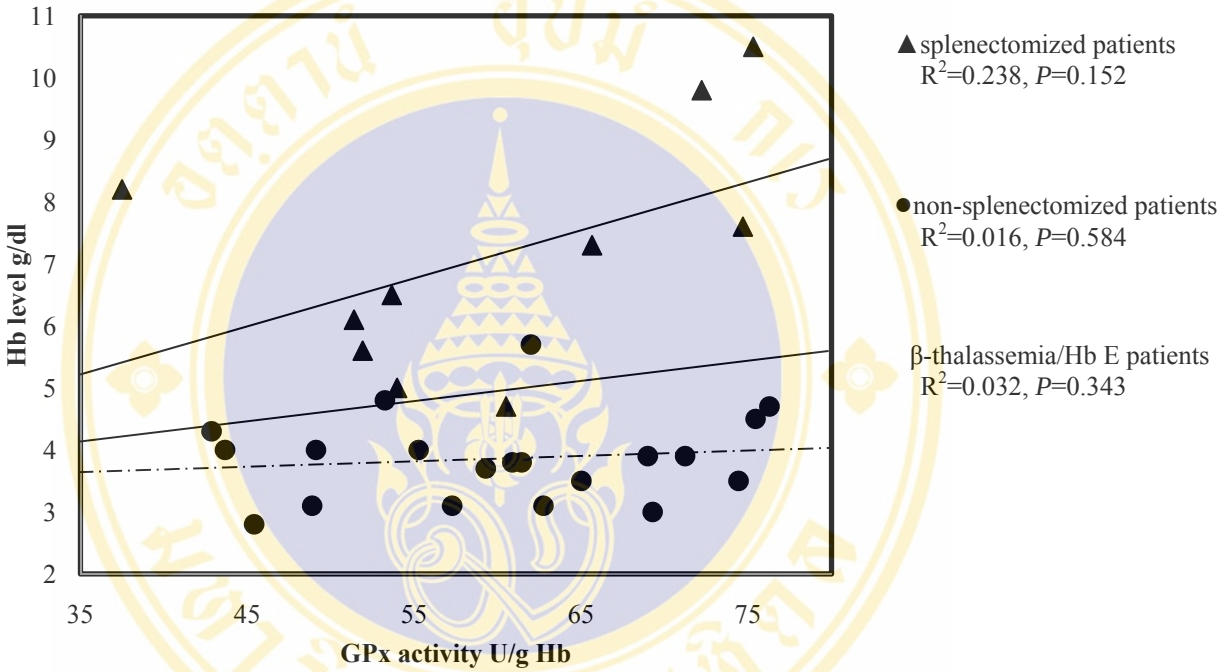


Figure 36 Correlation between hemoglobin (Hb) level and glutathione peroxidase (GPX) activity in β-thalassemia/Hb E patients (▲ = splenectomized patients, and ● = non-splenectomized patients).

8. Plasma protein carbonyl level in normal subjects and β-thalassemia/Hb E patients

The plasma protein carbonyl level was represented as mean ± SEM. Plasma protein carbonyl content in normal subjects was shown in Table 20. The protein carbonyl contents in males (23.33 + 1.77 nmol/ml or 1.48 ± 0.14 nmol/mg protein) were not significantly different from females (21.12 + 1.48 nmol/ml or 1.72 ± 0.15 nmol/mg protein) in normal subjects (p=0.452, 0.273) (Table 21). The average of plasma protein carbonyl in normal subjects was 22.09 ± 1.13 nmol/ml or 1.62 ± 0.10 nmol/mg protein.

The plasma protein carbonyl level in SP and NS β-thalassemia/Hb E patients were shown in Tables 22 and 23, respectively. The protein carbonyl between male and female samples was not statistically significant difference in SP and NS patients (p=0.933 and 0.775, respectively). The plasma protein carbonyl was significant different between sex in SP patients when data expressed as nmol/mg protein (p=0.042) (Table 24). The plasma protein carbonyl level in SP patients were 28.82 ± 3.47 nmol/ml or 2.19 ± 0.26 nmol/mg protein which were significantly higher than in NS patients 21.48 ± 1.46 nmol/ml or 1.60 ± 0.14 nmol/mg protein (p=0.03 and 0.021, respectively) (Table 25). The plasma protein carbonyl in patients was 23.82 ± 0.98 nmol/ml or 1.71 ± 0.13 nmol/mg protein.

The comparison of plasma carbonyl level in normal subjects and β-thalassemia/Hb E patients were shown in Table 25. Plasma protein carbonyl in normal subjects was 22.09 ± 1.13 nmol/ml which was not statistically significant difference from β-thalassemia/Hb E patients (23.82 ± 0.98, p=0.749). However, the significant difference was found in normal subjects and SP β-thalassemia/Hb E patients. The protein carbonyl level in SP patients was 28.82 ± 3.47 nmol/ml and higher than normal subjects (p=0.030). The results were comparable when expressed as nmol/mg protein (p=0.021) (Table 26, Figure 37).

Table 20 Plasma protein carbonyl level in normal subjects

Name	Sex	Age	Plasma Protein carbonyl	
			nmol/ml	nmol/mg protein
CHT	M	24	34.33	3.10
SAI	M	38	13.24	0.99
NAT	M	20	10.48	0.76
PAI	M	24	20.95	1.52
PIS	M	24	16.66	1.08
KRT	M	24	23.59	1.46
ADS	M	26	23.48	1.14
CHP	M	49	30.98	1.73
PRH	M	22	24.10	1.58
AEP	M	23	49.98	3.62
TNS	M	24	22.74	1.65
KOM	M	24	25.52	1.85
SRV	M	24	20.98	1.52
WTC	M	24	36.52	2.57
Mean $\pm$ SEM		(n=14)	23.33 $\pm$ 1.77	1.48 $\pm$ 0.14
RTY	F	40	17.65	1.09
SUP	F	29	22.13	2.07
PCG	F	24	21.90	1.64
AUK	F	36	29.61	2.73
PAN	F	24	22.89	1.73
KNT	F	24	19.70	1.26
BSP	F	26	14.30	1.04
WAP	F	50	16.50	1.27
SUG	F	22	25.79	1.94
PRA	F	39	16.80	1.27
ARP	F	24	28.19	2.18
SUK	F	22	19.36	1.23
SUC	F	23	28.89	2.08
PAP	F	24	11.98	0.93
SKN	F	24	34.45	2.57
TNP	F	35	35.49	2.49
Mean $\pm$ SEM		(n=16)	21.12 $\pm$ 1.48	1.72 $\pm$ 0.15

Table 21 Comparison of plasma protein carbonyl level in normal subjects between males and females

Subjects		Plasma Protein carbonyl	
		nmol/ml	**nmol/mg protein**
Normal subjects			
male	(n=14)	23.33 ± 1.77	1.48 ± 0.14
female	(n=16)	21.12 ± 1.48	1.72 ± 0.15
p-value		0.452	0.273
Total	(n=30)	22.09 ± 1.13	1.62 ± 0.10

*Values were given as mean ± SEM

Table 22 Plasma protein carbonyl level in splenectomized β-thalassemia/Hb E patients

Name	Sex	Age	Type	Plasma Protein carbonyl	
				nmol/ml	
NPJ	M	42	SP	13.41	0.96
APR	M	25	SP	28.03	1.09
NPN	M	22	SP	19.23	2.01
PJJ	M	30	SP	17.05	0.75
GRJ	M	20	SP	35.70	2.49
TYT	M	28	SP	45.36	1.64
Mean±SEM		(n=6)		29.07 ± 5.25	1.81 ± 0.30
VPK	F	21	SP	35.83	2.49
PLP	F	17	SP	14.50	1.59
CTM	F	30	SP	29.05	3.12
APT	F	44	SP	20.30	3.09
Mean ± SEM		(n=4)		28.39 ± 4.49	2.57 ± 0.72
Total mean		(n=10)		28.82 ± 3.47	2.19 ± 0.26

Table 23 Plasma protein carbonyl level in non-splenectomized β-thalassemia/Hb E patients

Name	Sex	Age	Type	Plasma Protein carbonyl	
				nmol/ml	nmol/mg protein
CNB	M	45	NS	19.91	1.52
SRP	M	52	NS	17.55	2.02
VSB	M	16	NS	26.92	2.85
TSC	M	19	NS	13.64	0.97
DNN	M	18	NS	19.91	1.52
SPS	M	41	NS	17.55	0.95
TNK	M	39	NS	26.92	1.96
SYU	M	27	NS	13.64	1.32
Mean ± SEM			(n=8)	20.34 ± 1.87	1.64 ± 0.22
CNP	F	22	NS	21.41	1.02
MNR	F	31	NS	21.39	1.90
YSN	F	54	NS	21.09	1.08
GST	F	30	NS	10.72	1.02
YPN	F	21	NS	20.73	1.00
JTJ	F	35	NS	11.35	1.32
BSP	F	49	NS	20.39	1.42
PGS	F	24	NS	12.14	0.93
APP	F	24	NS	15.69	1.59
PTN	F	28	NS	22.78	2.15
VTY	F	27	NS	31.27	2.16
VPJ	F	34	NS	35.83	3.20
Mean ± SEM			(n=12)	22.27 ± 2.16	1.57 ± 0.20
Total mean			(n=20)	21.48 ± 1.46	1.60 ± 0.14

Table 24 Comparison of plasma protein carbonyl level in SP and NS β-thalassemia/Hb E patients between males and females

Subjects		Plasma Protein carbonyl*	
		nmol/ml	nmol/mg protein
β-thalassemia/Hb E Splenectomized			
Male	(n=6)	29.07 ± 5.25	1.81 ± 0.30
Female	(n=4)	28.39 ± 4.49	2.57 ± 0.72
p-value		0.933	0.042
Total	(n=10)	28.82 ± 3.47	2.19 ± 0.26
β-thalassemia/Hb E Non-splenectomized			
Male	(n=8)	20.34 ± 1.87	1.64 ± 0.22
Female	(n=12)	22.27 ± 2.16	1.57 ± 0.20
p-value		0.775	0.816
Total	(n=20)	21.48 ± 1.46	1.60 ± 0.14
β-thalassemia/Hb E	(n=30)	23.82 ± 0.98	1.77 ± 0.13

*Values were given as mean ± SEM

Table 25 Comparison of plasma protein carbonyl level in normal subjects and β-thalassemia/Hb E patients

Subjects	Plasma Protein carbonyl*	
	nmol/ml	nmol/mg protein
Normal control (n=30)	22.09 ± 1.13	1.62 ± 0.10
β-thalassemia/Hb E (n=30)	23.82 ± 0.98	1.77 ± 0.13
p-value	0.749	0.611
Splenectomized (n=10)	28.82 ± 3.47	2.19 ± 0.26
Non-splenectomized (n=20)	21.48 ± 1.46	1.60 ± 0.14
p-value	0.030	0.021

*Values were given as mean ± SEM

Table 26 Comparison of plasma protein carbonyl level in normal subjects and SP β-thalassemia/Hb E patients

Subjects	Plasma Protein carbonyl*	
	nmol/ml	nmol/mg protein
Normal control (n=30)	22.09 ± 1.13	1.62 ± 0.10
Splenectomized (n=10)	28.82 ± 3.47	2.19 ± 0.26
p-value	0.021	0.407

*Values were given as mean ± SEM

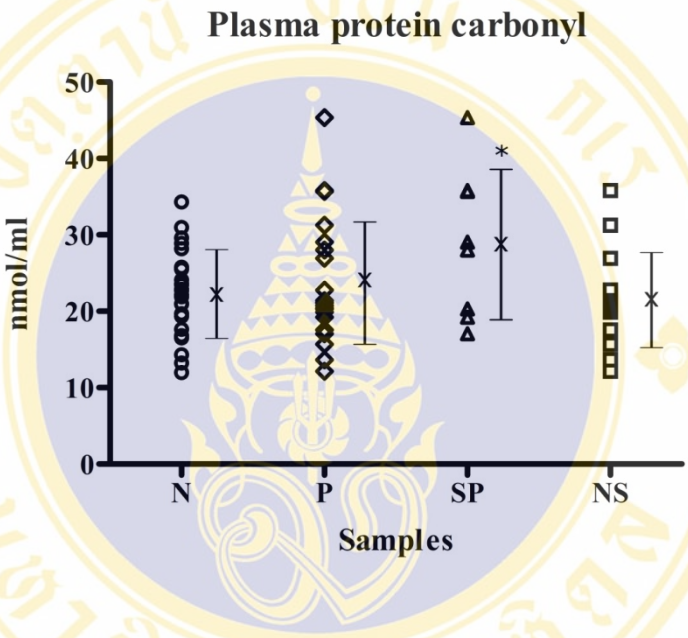


Figure 37 Scattergram of protein carbonyl in plasma samples of normal subjects (**o**), β-thalassemia/Hb E (n=30) (◊), splenectomized β-thalassemia/Hb E (n=10) (Δ), and non-splenectomized β-thalassemia/Hb E (n=20) (□). This result is shown as the mean ± SD. * Significantly different from normal subjects with $p<0.05$.

CHAPTER V

DISCUSSION

An oxidant injury by oxidative stress was observed in β-thalassemia disease. The elevation of oxidative stress in thalassemia is caused by their pathophysiology of disease. The mutation on globin genes lead to imbalanced globin chain synthesis. While the β-globin chain in thalassemia is reduced, the α-globin chain continues to synthesize at normal level resulting in the accumulation of α-globin chain in erythroid precursors. The oxidative injury is caused by ROS which are generated in β-thalassemia by excess of unmatched globin chain. The precipitation of α-globin chains in erythroid precursor cells lead to forming of inclusion body. Oxidation on lipid membrane by ROS gives a result in red cell damage. Releasing heme and free iron from red cell brings cell to oxidative damages (12) (39). Excess of α-globin chains bind to membrane protein and alter their structure and function. Membrane-bound and heme-containing compounds and band-3 aggregates were constantly presented in thalassemic RBC (141). Heme and free iron are sources of free radicals that can damage several red cell components and various kinds of biomolecules (34, 142, 143). The ineffective erythropoidesis, hemolysis of mature red blood cells, hypochromic, and microcytic red cells lead to anemia in thalassemic patients. Low level of hemoglobin in β-thalassemia/Hb E patients was observed in this study (7.07 ± 0.27 g/dl) (Table 7). Hydroxyl radical was abnormally generated high content in the β-thalassemic red blood cells (7). Furthermore, the ROS species attacked biological lipid membrane which subsequently made cell damage. In addition, the accumulation of free iron from hemolysis and iron absorption was a major source of free radical or ROS generation. The production of activated oxygen radicals from Fenton's reaction (ferrous iron with oxygen) was accelerated in patient more than normal subjects (144). The ROS did not only affect on biological lipid membrane but also changed hemoglobin to methemoglobin.

There were many studies suggesting that thalassemic patients were under oxidative stress because of misbalancing of oxidants and antioxidants (12, 48, 143). The high level of serum iron, free iron, ferritin, and NTBI has been observed in thalassemic patients (12, 16, and 145). The acute iron overload was found in β-thalassemia leading to NTBI accumulation. Furthermore, NTBI contributed to generate free radicals in Fenton's reaction (146). There were many oxidative products that were used as an oxidative marker in many diseases. The most common marker of lipid oxidation in thalassemia is MDA. Plasma MDA level was studied in thalassemic patients and found to be higher than normal controls (42, 44). However, the free radicals were not only attacked lipid molecule but also protein. ROS-mediated oxidation of proteins leads to the formation of reactive carbonyl compounds. In this study, protein carbonyl was specifically reacted with DNPH in spectrophotometry technique. The level of modified protein can be quantified by measuring carbonyl content which increased in many diseases.

Several authors demonstrated high level of oxidative modification protein in term of carbonyl contents in various disease such as inflammatory bowel disease, arthritis, chronic lung disease, chronic renal failure, diabetes, and sepsis (42) (147) (98) (148). The lesion of protein carbonyl was found in brain of Alzheimer's disease when stained with immune reactivity (147). We found a non statistically difference in plasma protein between β-thalassemia/Hb E patients and normal subjects the same as previous study (42). The SP β-thalasemia/Hb E patients contained plasma protein carbonyl level higher than NS and normal subjects. The high levels of carbonyl compounds reflected the severity of diseases. Trombetta *et. al.,* (148) found the increased serum protein carbonyl level in patients with β-thalassemia major. The elevation of carbonyl groups may be correlated with the progression and severity of the disease.

The increase of carbonyl groups was caused by unbalanced of homeostasis between oxidative products and antioxidant status in patients (21). In SP β-thalassemia/Hb E patients may be suffered from oxidative stress as in β-thalassemia major. Protein carbonyl does not depend on sex but depending on pathophysiology of diseases. Under protein modification by oxidative stress, oxidized proteins were

degraded by protease. The active 4-hydroxynonenal (4-HNE), lipid peroxidation product, reacted with histidine (His), cysteine (Cys), and lysine (Lys) residues on protein side chain to produce modified proteins. These proteins were not easily degraded by proteasome. Some carbonyl reactive compounds such as 4-HNE was also able to inhibit protease in proteasome (149).

The investigation of erythrocyte glutathione-related enzyme and whole blood glutathione concentration were used to describe the metabolism of glutathione in β-thalassemia/Hb E disease. Glutathione was defined as an essential thiol molecule that used to prevent and against oxidative stress. Inactivation and removal of ROS depend on reactions that involved in the antioxidant defense system. There were many antioxidant enzymes in erythrocytes such as SOD, CAT, GPX, GR, and GST, which were related to human diseases (150).

We investigated the activity of glutathione-related enzyme in RBC of thalassemic patients. In agreement with Kalpravidh *et. al.,* (151), our results showed the high level of GST, GPX and GR activity in erythrocytes. Plasma and blood GST have been studied in various conditions such as non-alcoholic steatohepatitis (25), lung malignancy (152), colorectal cancer (153), hepatocellular damage (154), and carcinoma cells (155). The activity of GST in these diseases was higher than normal controls. In this study, GST activity was 2.3-fold higher than normal subjects. The GST was used to detoxify many drug metabolites and including free radicals. A conjugation of GSH with free radical or metabolite was catalyzed by GST leading to mercapturic acid production (122). The high level of blood GST activity was induced by several pro-oxidants (156). Interestingly, GST activity was increased during development of adaptively detoxified toxic carbonyl-, peroxide-, and epoxide-containing metabolites products (157). Glutathione peroxidase was overwhelmed to completely remove of H_2O_2, since the K_m of GPX was able to function at low concentration of H_2O_2 (48). The activity of GPX in thalassemia patients was increased 6-folds when compared with normal subjects. We found high activity of GPX in these thalassemic patients as previous studies (14, 16, and 151). Furthermore, the high activity of GPX was also found in other diseases such as type-1 diabetes (158), cancer (159), and rheumatoid arthritis (160). It was used to protect against ROS in humans

especially H_2O_2. The enzymatic antioxidants (GST and GPX) scavenged free radicals or ROS, GSH was used to conjugate with reactive species or act as proton donor. The GSH was converted to GSSG during this process. Glutathione reductase was used in homeostasis of glutathione level in the cells. This enzyme was able to reverse GSSG to GSH. The β-thalassemia/Hb E patients contained higher activity of GR than normal subjects. This GR activity in β-thalassemia/Hb E patients was contributed to regeneration of GSH. The catalytic of GR in glutathione redox cycle was useful to conserve intracellular GSH (11, 30) Furthermore, indirect conversion of GSSG to GSH was coupled with glucose-6-phosephase dehydrogenase (G6PD) which was increased the same as GR activity in β-thalassemia/Hb E patients (16).

The glutathione is highly abundant in the cytosol, nuclei, and mitochondria (161). In living cells, glutathione is present mainly in a reduced form (GSH), whereas oxidized form (GSSG) is detected at low levels (102). The most crucial role of glutathione is to scavenge free radicals or ROS. Many studies have been suggested that GSH plays a critical role in protecting the living cells from toxicity and diseases. The blood GSH concentrations may serve as an indicator for human diseases. In this study, the highly sensitivity and selectivity of HPLC was used to measure the glutathione level in whole blood samples. Recently, the HPLC technique was used to measure glutathione and related thiols in biological samples because of short period of incubation and elution time. The specific conjugations of GSH and bromobimane fluorescence were used to determine total and oxidized glutathione. The whole blood samples were measured glutathione level under acidification solution for preventing of GSH auto-oxidation. In our study, glutathione was measured as total and GSSG in whole blood samples. While total glutathione (tGSH) was composed of GSH and GSSG, GSH was calculated from the subtraction of tGSH and GSSG. The total glutathione level was measured in the term of GSH and GSSG during conversion of GSSG to GSH by power reducing reagent (DTT). The GSSG level was analyzed under excess of NEM to prevent artificial GSSG from auto-oxidation of GSH (18, 162, 163). The method analyzing glutathione was modified from previous studies (140) shown in Figure 19, the peak of glutathione was distinctly separated with good resolution. Under our condition, the glutathione peak was eluted after GSH standard

as previous study (164). The level of tGSH, GSH and GSSG in normal subjects were 838.22 ± 27.59, 574.33 ± 20.97, and 263.89 ± 17.24 μmol/ml, respectively. Our results were in good agreement with previous study, which tGSH and GSH level in healthy subjects were 941 ± 155 and 849 ± 163 μmol/ml, respectively (165). The significant difference was observed in males and females of normal subjects in this study whereas there were not different in both groups of patients. The β-thalassemia/Hb E patients contained lower level of tGSH and GSH than normal subjects. The observation of low level of GSH reflected from human diseases and life habitat (18, 161). Under oxidative stress, β-thalassemia/Hb E patients used high amount of GSH to protect against ROS. An increase in GSSG was observed in this study when compared to normal subjects. This may be due to the response from ROS. High level of GSSG was also shown in human diseases such as ischemia reperfusion (166), AIDS and diabetes (18). The redox status of GSH/GSSG was found to be significantly different between normal subjects and β-thalassemia/Hb E patients. The high level of GSSG led to decrease redox ratio in patients as in various human diseases (18). An increase in GSSG was associated with a significantly decrease in redox ratio in these patients suggesting they underwent oxidative stress.

The glutathione metabolism in β-thalassemia/Hb E patients was different from normal subjects. Our results showed the evidence of oxidative stress and high activity of glutathione related enzyme in good agreement with previous study (13). In the enzymatic antioxidant pathways (Figure 38), GSH was used as a co-substrate of GST and GPX. During an increase in antioxidant enzymes, GSH was used and led to decrease intracellular GSH. The previous study showed high activity of G6PD in β-thalassemia/Hb E patients which were used to conserve intracellular GSH (13). In this study, G6PD deficiency was not found in β-thalassemia/Hb E patients and normal subjects. The low of GSH level in β-thalassemia/Hb E patients was affected from their pathophysiology. The intracellular GSH was maintained by action of GR, recycling pathway, and food supply.

Although, intracellular GSH was supplied from food or obtained from redox cycle but it was not enough for maintain GSH level in these patients. In normal circumstance, glutathione was used in normal healthy subjects to protect against ROS

from metabolite, drug, chemical compounds and environmental pollutants. Furthermore, GSH was involved in many mechanisms such as conversion of inactive to active form of ascorbic acid (167), regulation of cell apoptosis (168), and maintenance thiol group in the cell (135). Our finding showed protein oxidation only in SP β-thalassemia/Hb E patients. High levels of enzymatic antioxidants (GST and GPX) indicated that patients were under oxidative stress. The low of GSH was indicated all of patients tend to eliminate the oxidant products and ROS. The redox ratio showed an inadequate of GSH in antioxidant defense system in patients. During oxidative stress, human cells try to decrease oxidative stress by increasing enzymatic antioxidant enzyme. Glutathione related enzyme was response to oxidative stress in the thalassemic patients. The capacity of the oxidation-reduction cycle may be related to the cellular content of GSH and GR. In particular, substantial evidence implicates GSH metabolism as a major determinant of thalassemia as therapeutic efficacy (169). The data implied that cellular antioxidant defense by GSH pathways in the patients were also useful and should be mentioned during therapeutic treatment. Under oxidative stress, the β-thalassemia/Hb E patients were used GSH as defense mechanism.

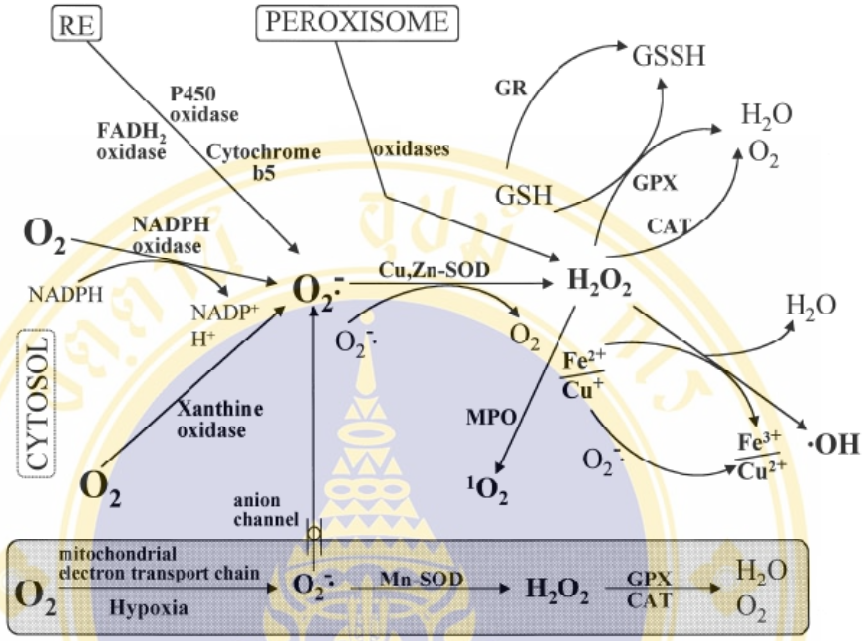


Figure 38 Generation of reactive oxygen species and the defense mechanisms against damage by active oxygen (150).

REFERENCES

1. Lichtman MA, Kioos TJ, Kaushansky K, Beutler E, Seligsohn U, Prchal JT. Williams Hematology. 7th ed. USA: McGeaw-Hill companies, Inc. 2006.
2. Olivieri NF. The beta-thalassemias. The New England journal of medicine. 1999;341(2):99-109.
3. Rund D, Rachmilewitz E. Beta-thalassemia. The New England journal of medicine. 2005;353(11):1135-46.
4. Sonakul D., Vicha S. Pathology of thalassemic diseases. Thailand: Amarin printing group 1986:20-3.
5. Fucharoen G, Fucharoen S, Sanchaisuriya K, Sae-Ung N, Suyasunanond U, Sriwilai P, et al. Frequency Distribution and Haplotypic Heterogeneity of beta(E)-Globin Gene among Eight Minority Groups of Northeast Thailand. Human heredity. 2002;53(1):18-22.
6. Wasi P, Na-Nakorn S, Pootrakul S, Sookanek M, Disthasongchan P, Panich V, et al. Alpha- and beta-thalassemia in Thailand. Annals of the New York Academy of Sciences. 1969;165(1):60-82.
7. Grinberg LN, Rachmilewitz EA, Kitrossky N, Chevion M. Hydroxyl radical generation in beta-thalassemic red blood cells. Free Radic Biol Med. 1995;18(3):611-5.
8. Livrea MA, Tesoriere L, Pintaudi AM, Calabrese A, Maggio A, Freisleben HJ, et al. Oxidative stress and antioxidant status in beta-thalassemia major: iron overload and depletion of lipid-soluble antioxidants. Blood. 1996;88(9):3608-14.
9. Bayir H. Reactive oxygen species. Crit Care Med. 2005;33(12 Suppl):S498-501.
10. Offer T, Bhagat A, Lal A, Atamna W, Singer ST, Vichinsky EP, et al. Measuring chromosome breaks in patients with thalassemia. Ann N Y Acad Sci. 2005;1054:439-44.

11. Denisov ET, Afanas'ev IB. Oxidation and Antioxidants in Organic Chemistry and Biology. USA: CRC; Har/Cdr edition 2005.
12. Schrier SL. Pathophysiology of thalassemia. Curr Opin Hematol. 2002;9(2):123-6.
13. Carpino A, Tarantino P, Rago V, De Sanctis V, Siciliano L. Antioxidant capacity in seminal plasma of transfusion-dependent beta-thalassemic patients. Exp Clin Endocrinol Diabetes. 2004;112(3):131-4.
14. Kassab-Chekir A, Laradi S, Ferchichi S, Haj Khelil A, Feki M, Amri F, et al. Oxidant, antioxidant status and metabolic data in patients with beta-thalassemia. Clinica chimica acta; international journal of clinical chemistry. 2003;338(1-2):79-86.
15. Anastassopoulou J, Anifantakis B, Anifantakis ZA, Dovas A, Theophanides T. The role of free radical reactions with haemoglobin and thalassaemia. Journal of inorganic biochemistry. 2000;79(1-4):327-9.
16. Chakraborty D, Bhattacharyya M. Antioxidant defense status of red blood cells of patients with beta-thalassemia and Ebeta-thalassemia. Clinica chimica acta; international journal of clinical chemistry. 2001;305(1-2):123-9.
17. Rachmilewitz EA, Kornberg A, Acker M. Vitamin E deficiency due to increased consumption in beta-thalassemia and in Gaucher's disease. Ann N Y Acad Sci. 1982;393:336-47.
18. Pastore A, Federici G, Bertini E, Piemonte F. Analysis of glutathione: implication in redox and detoxification. Clinica chimica acta; international journal of clinical chemistry. 2003;333(1):19-39.
19. Burdon CR-ERH. Free radical damage and its control: Amsterdam ; New York : Elsevier 1994.
20. Whalen R, Boyer TD. Human glutathione S-transferases. Semin Liver Dis. 1998;18(4):345-58.
21. Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R. Protein carbonyl groups as biomarkers of oxidative stress. Clin Chim Acta. 2003;329(1-2):23-38.
22. Berlett BS, Stadtman ER. Protein oxidation in aging, disease, and oxidative stress. The Journal of biological chemistry. 1997;272(33):20313-6.

23. Thelwall PE, Yemin AY, Gillian TL, Simpson NE, Kasibhatla MS, Rabbani ZN, et al. Noninvasive in vivo detection of glutathione metabolism in tumors. Cancer Res. 2005;65(22):10149-53.
24. Maher P. The effects of stress and aging on glutathione metabolism. Ageing Res Rev. 2005;4(2):288-314.
25. Nobili V, Pastore A, Gaeta LM, Tozzi G, Comparcola D, Sartorelli MR, et al. Glutathione metabolism and antioxidant enzymes in patients affected by nonalcoholic steatohepatitis. Clin Chim Acta. 2005;355(1-2):105-11.
26. Liu H, Harrell LE, Shenvi S, Hagen T, Liu RM. Gender differences in glutathione metabolism in Alzheimer's disease. J Neurosci Res. 2005;79(6):861-7.
27. Aukrust P, Muller F, Svardal AM, Ueland T, Berge RK, Froland SS. Disturbed glutathione metabolism and decreased antioxidant levels in human immunodeficiency virus-infected patients during highly active antiretroviral therapy--potential immunomodulatory effects of antioxidants. J Infect Dis. 2003;188(2):232-8.
28. Pastore A, Tozzi G, Gaeta LM, Giannotti A, Bertini E, Federici G, et al. Glutathione metabolism and antioxidant enzymes in children with Down syndrome. J Pediatr. 2003;142(5):583-5.
29. Ernest Beutler M.D., Marshall A. Lichtman M.D., Barry S. Coller M.D., Thomas J. Kipps M.D. Ph.D. HEMATOLOGY IN THE AGED. In: M.D. US, ed. *Williams Hematology*. 6 ed. USA: McGraw-Hill 2000:106-17.
30. Metzler DE. Biochemistry The Chemical Reactions of living cells. second ed 2002.
31. Voet D, Voet JG. Hemoglobin: Protein Function in microcosm. *Biochemistry*. 3 ed. U.S.A.: John Wiley and Sons, Inc. 2004:327-38.
32. Mader SS. Circulation. *Understanding Human Anatomy & Physiology*. 5 ed. USA: McGraw-Hill Science Engineering 2004:375-9.
33. Weatherall DJ, Provan AB. Red cells I: inherited anaemias. Lancet. 2000;355(9210):1169-75.
34. Smith C, Marks A, Lieberman M. Oxygen Toxicity and Free Radical Injury. *Marks' Basic Medical Biochemistry: A Clinical Approach*. 2 ed. USA: Lippincott Williams & Wilkins 2004:439-56.

35. Rees DC, Styles L, Vichinsky EP, Clegg JB, Weatherall DJ. The hemoglobin E syndromes. Annals of the New York Academy of Sciences. 1998;850:334-43.
36. Rees DC, Clegg JB, Weatherall DJ. Is hemoglobin instability important in the interaction between hemoglobin E and beta thalassemia? Blood. 1998;92(6):2141-6.
37. Sanchaisuriya K, Fucharoen S, Ratanasiri T, Sanchaisuriya P, Fucharoen G, Dietz E, et al. Thalassemia and hemoglobinopathies rather than iron deficiency are major causes of pregnancy-related anemia in northeast Thailand. Blood cells, molecules & diseases. 2006;37(1):8-11.
38. Weatherall DJ. Introduction to the problem of hemoglobin E-beta thalassemia. J Pediatr Hematol Oncol. 2000;22(6):551.
39. Eldor A, Rachmilewitz EA. The hypercoagulable state in thalassemia. Blood. 2002;99(1):36-43.
40. Datta P, Chakrabarty SB, Chakrabarty A, Chakrabarti A. Interaction of erythroid spectrin with hemoglobin variants: implications in beta-thalassemia. Blood cells, molecules & diseases. 2003;30(3):248-53.
41. Datta P, Basu S, Chakravarty SB, Chakravarty A, Banerjee D, Chandra S, et al. Enhanced oxidative cross-linking of hemoglobin E with spectrin and loss of erythrocyte membrane asymmetry in hemoglobin Ebeta-thalassemia. Blood cells, molecules & diseases. 2006;37(2):77-81.
42. Walter PB, Fung EB, Killilea DW, Jiang Q, Hudes M, Madden J, et al. Oxidative stress and inflammation in iron-overloaded patients with beta-thalassaemia or sickle cell disease. British journal of haematology. 2006;135(2):254-63.
43. Meral A, Tuncel P, Surmen-Gur E, Ozbek R, Ozturk E, Gunay U. Lipid peroxidation and antioxidant status in beta-thalassemia. Pediatric hematology and oncology. 2000;17(8):687-93.
44. Cighetti G, Duca L, Bortone L, Sala S, Nava I, Fiorelli G, et al. Oxidative status and malondialdehyde in beta-thalassaemia patients. European journal of clinical investigation. 2002;32 Suppl 1:55-60.

45. Şimşek F, Öztürk G, Kemahlı S, Erbaş D, Hasanoğlu A. Oxidant and antioxidant status in beta thalassemia major patients. Ankara Üniversitesi Tıp Fakültesi Mecmuası. 2005;58:34-8.
46. Tavazzi D, Duca L, Graziadei G, Comino A, Fiorelli G, Cappellini MD. Membrane-bound iron contributes to oxidative damage of beta-thalassaemia intermedia erythrocytes. British journal of haematology. 2001;112(1):48-50.
47. Aessopos A, Farmakis D, Deftereos S, Tsironi M, Tassiopoulos S, Moyssakis I, et al. Thalassemia heart disease: a comparative evaluation of thalassemia major and thalassemia intermedia. Chest. 2005;127(5):1523-30.
48. Rice-Evans CA, Burdon RH. Free Radical Damage and its Control. USA: Elsevier Science 1994.
49. Koppenol WH. NAMES FOR INORGANIC RADICALS (IUPAC Recommendations 2000). Pure Appl Chem. 2000;72(3):437–46.
50. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. The international journal of biochemistry & cell biology. 2007;39(1):44-84.
51. Fang YZ, Yang S, Wu G. Free radicals, antioxidants, and nutrition. Nutrition (Burbank, Los Angeles County, Calif. 2002;18(10):872-9.
52. Ohnishi ST, Ohnishi T, Muranaka S, Fujita H, Kimura H, Uemura K, et al. A possible site of superoxide generation in the complex I segment of rat heart mitochondria. Journal of bioenergetics and biomembranes. 2005;37(1):1-15.
53. Grivennikova VG, Vinogradov AD. Generation of superoxide by the mitochondrial Complex I. Biochimica et biophysica acta. 2006;1757(5-6):553-61.
54. Darley-Usmar V. The powerhouse takes control of the cell; the role of mitochondria in signal transduction. Free radical biology & medicine. 2004;37(6):753-4.
55. Turrens JF. Mitochondrial formation of reactive oxygen species. The Journal of physiology. 2003;552(Pt 2):335-44.

56. Durant R, Klouche K, Delbosc S, Morena M, Amigues L, Beraud JJ, et al. Superoxide anion overproduction in sepsis: effects of vitamin e and simvastatin. Shock (Augusta, Ga. 2004;22(1):34-9.
57. Gibson A, Lilley E. Superoxide anions, free-radical scavengers, and nitrergic neurotransmission. General pharmacology. 1997;28(4):489-93.
58. Liochev SI, Fridovich I. The effects of superoxide dismutase on H(2)O(2) formation. Free radical biology & medicine. 2007;42(10):1465-9.
59. Halliwell B, Clement MV, Long LH. Hydrogen peroxide in the human body. FEBS letters. 2000;486(1):10-3.
60. Spickett CM, Jerlich A, Panasenko OM, Arnhold J, Pitt AR, Stelmaszynska T, et al. The reactions of hypochlorous acid, the reactive oxygen species produced by myeloperoxidase, with lipids. Acta biochimica Polonica. 2000;47(4):889-99.
61. Pullar JM, Vissers MC, Winterbourn CC. Living with a killer: the effects of hypochlorous acid on mammalian cells. IUBMB life. 2000;50(4-5):259-66.
62. Coleman LG, Jr., Polanowska-Grabowska RK, Marcinkiewicz M, Gear AR. LDL oxidized by hypochlorous acid causes irreversible platelet aggregation when combined with low levels of ADP, thrombin, epinephrine, or macrophage-derived chemokine (CCL22). Blood. 2004;104(2):380-9.
63. Winterbourn CC. Biological reactivity and biomarkers of the neutrophil oxidant, hypochlorous acid. Toxicology. 2002;181-182:223-7.
64. Lloyd RV, Hanna PM, Mason RP. The origin of the hydroxyl radical oxygen in the Fenton reaction. Free radical biology & medicine. 1997;22(5):885-8.
65. Kehrer JP. The Haber-Weiss reaction and mechanisms of toxicity. Toxicology. 2000;149(1):43-50.
66. Cadet J, Delatour T, Douki T, Gasparutto D, Pouget JP, Ravanat JL, et al. Hydroxyl radicals and DNA base damage. Mutation research. 1999;424(1-2):9-21.
67. Berneburg M, Grether-Beck S, Kurten V, Ruzicka T, Briviba K, Sies H, et al. Singlet oxygen mediates the UVA-induced generation of the photoaging-

associated mitochondrial common deletion. The Journal of biological chemistry. 1999;274(22):15345-9.

68. Moan J, Juzenas P. Singlet oxygen in photosensitization. J Environ Pathol Toxicol Oncol. 2006;25(1-2):29-50.
69. King MM, Lai EK, McCay PB. Singlet oxygen production associated with enzyme-catalyzed lipid peroxidation in liver microsomes. The Journal of biological chemistry. 1975;250(16):6496-502.
70. Kanofsky JR, Hoogland H, Wever R, Weiss SJ. Singlet oxygen production by human eosinophils. The Journal of biological chemistry. 1988;263(20):9692-6.
71. Kanofsky JR, Sima P. Singlet oxygen production from the reactions of ozone with biological molecules. The Journal of biological chemistry. 1991;266(14):9039-42.
72. Rodrigues T, de Franca LP, Kawai C, de Faria PA, Mugnol KC, Braga FM, et al. Protective role of mitochondrial unsaturated lipids on the preservation of the apoptotic ability of cytochrome C exposed to singlet oxygen. The Journal of biological chemistry. 2007;282(35):25577-87.
73. Goosey JD, Zigler JS, Jr., Matheson IB, Kinoshita JH. Effects of singlet oxygen on human lens crystallins in vitro. Investigative ophthalmology & visual science. 1981;20(5):679-83.
74. Davies MJ. Reactive species formed on proteins exposed to singlet oxygen. Photochem Photobiol Sci. 2004;3(1):17-25.
75. Ravanat JL, Di Mascio P, Martinez GR, Medeiros MH, Cadet J. Singlet oxygen induces oxidation of cellular DNA. The Journal of biological chemistry. 2000;275(51):40601-4.
76. Girotti AW. Lipid hydroperoxide generation, turnover, and effector action in biological systems. Journal of lipid research. 1998;39(8):1529-42.
77. Babior BM. Phagocytes and oxidative stress. The American journal of medicine. 2000;109(1):33-44.
78. Ryter SW, Kim HP, Hoetzel A, Park JW, Nakahira K, Wang X, et al. Mechanisms of cell death in oxidative stress. Antioxidants & redox signaling. 2007;9(1):49-89.

79. Droge W. Free radicals in the physiological control of cell function. Physiological reviews. 2002;82(1):47-95.
80. Reynolds A, Laurie C, Lee Mosley R, Gendelman HE. Oxidative stress and the pathogenesis of neurodegenerative disorders. International review of neurobiology. 2007;82:297-325.
81. Emerit J, Edeas M, Bricaire F. Neurodegenerative diseases and oxidative stress. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie. 2004;58(1):39-46.
82. Strazzullo P, Puig JG. Uric acid and oxidative stress: relative impact on cardiovascular risk? Nutr Metab Cardiovasc Dis. 2007;17(6):409-14.
83. Kaneto H, Katakami N, Kawamori D, Miyatsuka T, Sakamoto K, Matsuoka TA, et al. Involvement of oxidative stress in the pathogenesis of diabetes. Antioxidants & redox signaling. 2007;9(3):355-66.
84. Pennathur S, Heinecke JW. Mechanisms for oxidative stress in diabetic cardiovascular disease. Antioxidants & redox signaling. 2007;9(7):955-69.
85. Gago-Dominguez M, Jiang X, Castelao JE. Lipid peroxidation, oxidative stress genes and dietary factors in breast cancer protection: a hypothesis. Breast Cancer Res. 2007;9(1):201.
86. Stadtman ER. Role of oxidant species in aging. Current medicinal chemistry. 2004;11(9):1105-12.
87. Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. Nature. 2000;408(6809):239-47.
88. Angelucci E, Brittenham GM, McLaren CE, Ripalti M, Baronciani D, Giardini C, et al. Hepatic iron concentration and total body iron stores in thalassemia major. The New England journal of medicine. 2000;343(5):327-31.
89. Scott MD. H2O2 injury in beta thalassemic erythrocytes: protective role of catalase and the prooxidant effects of GSH. Free radical biology & medicine. 2006;40(7):1264-72.
90. Dean RT, Fu S, Stocker R, Davies MJ. Biochemistry and pathology of radical-mediated protein oxidation. The Biochemical journal. 1997;324 (Pt 1):1-18.

91. Stadtman ER, Levine RL. Protein oxidation. Annals of the New York Academy of Sciences. 2000;899:191-208.
92. Schuessler H, Schilling K. Oxygen effect in the radiolysis of proteins. Part 2. Bovine serum albumin. International journal of radiation biology and related studies in physics, chemistry, and medicine. 1984;45(3):267-81.
93. Stadtman ER. Protein oxidation and aging. Science (New York, NY. 1992;257(5074):1220-4.
94. Stadtman ER. Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. Annual review of biochemistry. 1993;62:797-821.
95. Theodore E Brown, H. Eugene LeMay, Bursten BE. The Chemistry of Life: Organic and Biological Chemistry. In: Edition t, ed. *Chemistry: The Central Science [STUDENT EDITION]*: Prentice Hall 2005:1248 pages.
96. Otera J. Modern carbonyl chemistry. New York: Weinheim Wiley-VCH 2000.
97. Ellis EM. Reactive carbonyls and oxidative stress: Potential for therapeutic intervention. Pharmacology & therapeutics. 2007;115(1):13-24.
98. Dalle-Donne I, Giustarini D, Colombo R, Rossi R, Milzani A. Protein carbonylation in human diseases. Trends in molecular medicine. 2003;9(4):169-76.
99. Pantke U, Volk T, Schmutzler M, Kox WJ, Sitte N, Grune T. Oxidized proteins as a marker of oxidative stress during coronary heart surgery. Free radical biology & medicine. 1999;27(9-10):1080-6.
100. Cecarini V, Gee J, Fioretti E, Amici M, Angeletti M, Eleuteri AM, et al. Protein oxidation and cellular homeostasis: Emphasis on metabolism. Biochimica et biophysica acta. 2007;1773(2):93-104.
101. Wu G, Fang YZ, Yang S, Lupton JR, Turner ND. Glutathione metabolism and its implications for health. The Journal of nutrition. 2004;134(3):489-92.
102. Meister A, Anderson ME. Glutathione. Annual review of biochemistry. 1983;52:711-60.
103. Griffith OW. Biologic and pharmacologic regulation of mammalian glutathione synthesis. Free radical biology & medicine. 1999;27(9-10):922-35.

104. Lu SC. Regulation of hepatic glutathione synthesis: current concepts and controversies. Faseb J. 1999;13(10):1169-83.
105. Njalsson R. Glutathione synthetase deficiency. Cell Mol Life Sci. 2005;62(17):1938-45.
106. David L. Nelson (Author) MMCA. Lehninger Principles of Biochemistry. 4th ed. USA: W. H. Freeman 2004.
107. Brosnan JT, Brosnan ME. The sulfur-containing amino acids: an overview. The Journal of nutrition. 2006;136(6 Suppl):1636S-40S.
108. Lu SC. Regulation of hepatic glutathione synthesis. Seminars in liver disease. 1998;18(4):331-43.
109. Griffith OW, Meister A. Glutathione: interorgan translocation, turnover, and metabolism. Proceedings of the National Academy of Sciences of the United States of America. 1979;76(11):5606-10.
110. Griffith OW, Novogrodsky A, Meister A. Translocation of glutathione from lymphoid cells that have markedly different gamma-glutamyl transpeptidase activities. Proceedings of the National Academy of Sciences of the United States of America. 1979;76(5):2249-52.
111. Griffith OW, Meister A. Translocation of intracellular glutathione to membrane-bound gamma-glutamyl transpeptidase as a discrete step in the gamma-glutamyl cycle: glutathionuria after inhibition of transpeptidase. Proceedings of the National Academy of Sciences of the United States of America. 1979;76(1):268-72.
112. Anderson ME, Meister A. Transport and direct utilization of gamma-glutamylcyst(e)ine for glutathione synthesis. Proceedings of the National Academy of Sciences of the United States of America. 1983;80(3):707-11.
113. Meister A. Transport and metabolism of glutathione and gamma-glutamyl amino acids. Biochemical Society transactions. 1983;11(6):793-4.
114. Griffith OW, Bridges RJ, Meister A. Transport of gamma-glutamyl amino acids: role of glutathione and gamma-glutamyl transpeptidase. Proceedings of the National Academy of Sciences of the United States of America. 1979;76(12):6319-22.

115. Anderson ME, Meister A. Dynamic state of glutathione in blood plasma. The Journal of biological chemistry. 1980;255(20):9530-3.

116. Meister A. Glutathione, ascorbate, and cellular protection. Cancer research. 1994;54(7 Suppl):1969s-75s.

117. Kanno H. [gamma-Glutamyl cysteine synthetase deficiency]. Ryoikibetsu shokogun shirizu. 1998(18 Pt 1):371-2.

118. Wellner VP, Sekura R, Meister A, Larsson A. Glutathione synthetase deficiency, an inborn error of metabolism involving the gamma-glutamyl cycle in patients with 5-oxoprolinuria (pyroglutamic aciduria). Proceedings of the National Academy of Sciences of the United States of America. 1974;71(6):2505-9.

119. Kaplowitz N, Aw TY, Ookhtens M. The regulation of hepatic glutathione. Annual review of pharmacology and toxicology. 1985;25:715-44.

120. Hayes JD, Flanagan JU, Jowsey IR. Glutathione transferases. Annual review of pharmacology and toxicology. 2005;45:51-88.

121. Paolisso G, Di Maro G, Pizza G, D'Amore A, Sgambato S, Tesauro P, et al. Plasma GSH/GSSG affects glucose homeostasis in healthy subjects and non-insulin-dependent diabetics. The American journal of physiology. 1992;263(3 Pt 1):E435-40.

122. Coleman M. Human Drug Metabolism: An Introduction. England: John Wiley & Sons, Ltd. 2005.

123. van Bladeren PJ. Glutathione conjugation as a bioactivation reaction. Chemico-biological interactions. 2000;129(1-2):61-76.

124. Ioannides C. Glutathione S-transferases. In: Hayes PJSaJD, ed. *Current Toxicology Series: Enzyme Systems that Metabolise Drugs and Other Xenobiotics*. USA: John Wiley & Sons Inc 2002:588.

125. Flohe L. Glutathione peroxidase: fact and fiction. Ciba Foundation symposium. 1978(65):95-122.

126. Arthur JR. The glutathione peroxidases. Cell Mol Life Sci. 2000;57(13-14):1825-35.

127. Prabhakar R, Vreven T, Morokuma K, Musaev DG. Elucidation of the mechanism of selenoprotein glutathione peroxidase (GPx)-catalyzed

hydrogen peroxide reduction by two glutathione molecules: a density functional study. Biochemistry. 2005;44(35):11864-71.

128. Kamerbeek NM, van Zwieten R, de Boer M, Morren G, Vuil H, Bannink N, et al. Molecular basis of glutathione reductase deficiency in human blood cells. Blood. 2007;109(8):3560-6.

129. Kirsch M, De Groot H. NAD(P)H, a directly operating antioxidant? Faseb J. 2001;15(9):1569-74.

130. Kalyanaraman B. Thiyl radicals in biological systems: significant or trivial? Biochemical Society symposium. 1995;61:55-63.

131. Starke DW, Chock PB, Mieyal JJ. Glutathione-thiyl radical scavenging and transferase properties of human glutaredoxin (thioltransferase). Potential role in redox signal transduction. The Journal of biological chemistry. 2003;278(17):14607-13.

132. Dizdaroglu M, Jaruga P, Birincioglu M, Rodriguez H. Free radical-induced damage to DNA: mechanisms and measurement. Free radical biology & medicine. 2002;32(11):1102-15.

133. Sandstrom BE, Marklund SL. Effects of variation in glutathione peroxidase activity on DNA damage and cell survival in human cells exposed to hydrogen peroxide and t-butyl hydroperoxide. The Biochemical journal. 1990;271(1):17-23.

134. Sharma R, Awasthi S, Zimniak P, Awasthi YC. Transport of glutathione-conjugates in human erythrocytes. Acta biochimica Polonica. 2000;47(3):751-62.

135. Kidd PM. Glutathione: Systemic Protectant Against Oxidative and Free Radical Damage. Alternative Medicine Review. 1997;2(3):155-76.

136. Sonntag CV. Chemical Basis of Radiation Biology. USA: Taylor & Francis 1978.

137. Masella R, Di Benedetto R, Vari R, Filesi C, Giovannini C. Novel mechanisms of natural antioxidant compounds in biological systems: involvement of glutathione and glutathione-related enzymes. The Journal of nutritional biochemistry. 2005;16(10):577-86.

138. Habig WH, Pabst MJ, Jakoby WB. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. The Journal of biological chemistry. 1974;249(22):7130-9.

139. Andersen HR, Nielsen JB, Nielsen F, Grandjean P. Antioxidative enzyme activities in human erythrocytes. Clinical chemistry. 1997;43(4):562-8.

140. Pastore A, Massoud R, Motti C, Lo Russo A, Fucci G, Cortese C, et al. Fully automated assay for total homocysteine, cysteine, cysteinylglycine, glutathione, cysteamine, and 2-mercaptopropionylglycine in plasma and urine. Clinical chemistry. 1998;44(4):825-32.

141. Cappellini MD, Tavazzi D, Duca L, Graziadei G, Mannu F, Turrini F, et al. Metabolic indicators of oxidative stress correlate with haemichrome attachment to membrane, band 3 aggregation and erythrophagocytosis in beta-thalassaemia intermedia. British journal of haematology. 1999;104(3):504-12.

142. Molineux G, Foote M, Elliott SG. Erythropoietins and Erythropoiesis · Molecular, Cellular, Preclinical, and Clinical Biology. USA 2003.

143. Provan D, Singer CRJ, Baglin T, Lilleyman J. Oxford Handbook of Clinical Haematology. New York: Oxford University Press, Inc. 2004.

144. C.Mentzer W. The hereditary hemolytic anemia. USA: Churchill Livingstone 1989.

145. Dhawan V, Kumar Kh R, Marwaha RK, Ganguly NK. Antioxidant status in children with homozygous thalassemia. Indian pediatrics. 2005;42(11):1141-5.

146. Syed BA, Sargent PJ, Farnaud S, Evans RW. An overview of molecular aspects of iron metabolism. Hemoglobin. 2006;30(1):69-80.

147. Aksenov MY, Aksenova MV, Butterfield DA, Geddes JW, Markesbery WR. Protein oxidation in the brain in Alzheimer's disease. Neuroscience. 2001;103(2):373-83.

148. Trombetta D, Gangemi S, Saija A, Minciullo PL, Cimino F, Cristani M, et al. Increased protein carbonyl groups in the serum of patients affected by thalassemia major. Annals of hematology. 2006;85(8):520-2.

149. Negre-Salvayre A, Coatrieux C, Ingueneau C, Salvayre R. Advanced lipid peroxidation end products in oxidative damage to proteins. Potential role in diseases and therapeutic prospects for the inhibitors. Br J Pharmacol. 2007.

150. Mates JM, Perez-Gomez C, Nunez de Castro I. Antioxidant enzymes and human diseases. Clinical biochemistry. 1999;32(8):595-603.

151. Kalpravidh RW, Wichit A, Siritanaratkul N, Fucharoen S. Effect of coenzyme Q10 as an antioxidant in beta-thalassemia/Hb E patients. BioFactors. 2005;25:225-34.

152. K.B. Gupta ST, Vani Garg and Harbans Lai. Plasma glutathione-s-transferase activity in lung malignancy. Ind J Tub. 2000;47:227-8.

153. Nomani H, Ghobadloo SM, Yaghmaei B, Rezvanie NA, Yaghmaei K. Glutathione S-transferases activity in patients with colorectal cancer. Clin Biochem. 2005;38(7):621-4.

154. Kumtepe Y, Borekci B, Aksoy H, Altinkaynak K, Ingec M, Ozdiller O. Measurement of plasma glutathione S-transferase in hepatocellular damage in pre-eclampsia. J Int Med Res. 2002;30(5):483-7.

155. Ferruzzi E, Franceschini R, Cazzolato G, Geroni C, Fowst C, Pastorino U, et al. Blood glutathione as a surrogate marker of cancer tissue glutathione S-transferase activity in non-small cell lung cancer and squamous cell carcinoma of the head and neck. European Journal of Cancer. 2003;39(7):1019-29.

156. Hayes JD, McLellan LI. Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. Free radical research. 1999;31(4):273-300.

157. Hayes JD, Pulford DJ. The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Critical reviews in biochemistry and molecular biology. 1995;30(6):445-600.

158. Likidlilid A, Patchanans N, Poldee S, Peerapatdit T. Glutathione and glutathione peroxidase in type 1 diabetic patients. Journal of the Medical Association of Thailand = Chotmaihet thangphaet. 2007;90(9):1759-67.

159. Aggarwal S, Subberwal M, Kumar S, Sharma M. Brain tumor and role of beta-carotene, a-tocopherol, superoxide dismutase and glutathione peroxidase. Journal of cancer research and therapeutics. 2006;2(1):24-7.
160. Kalpakcioglu B, Senel K. The interrelation of glutathione reductase, catalase, glutathione peroxidase, superoxide dismutase, and glucose-6-phosphate in the pathogenesis of rheumatoid arthritis. Clin Rheumatol. 2007.
161. Camera E, Picardo M. Analytical methods to investigate glutathione and related compounds in biological and pathological processes. Journal of chromatography. 2002;781(1-2):181-206.
162. Wendell PL. Measurement of oxidized glutathione and total glutathione in the perfused rat heart. The Biochemical journal. 1970;117(4):661-5.
163. Serru V, Baudin B, Ziegler F, David JP, Cals MJ, Vaubourdolle M, et al. Quantification of reduced and oxidized glutathione in whole blood samples by capillary electrophoresis. Clinical chemistry. 2001;47(7):1321-4.
164. Pastore A, Piemonte F, Locatelli M, Lo Russo A, Gaeta LM, Tozzi G, et al. Determination of blood total, reduced, and oxidized glutathione in pediatric subjects. Clinical chemistry. 2001;47(8):1467-9.
165. Michelet F, Gueguen R, Leroy P, Wellman M, Nicolas A, Siest G. Blood and plasma glutathione measured in healthy subjects by HPLC: relation to sex, aging, biological variables, and life habits. Clinical chemistry. 1995;41(10):1509-17.
166. Katz A, Oldham KT, Guice KS, Coran AG. Oxidized glutathione as a marker of ischemia reperfusion associated with single lung transplantation. Journal of the American College of Surgeons. 1995;180(1):25-32.
167. Meister A. Glutathione-ascorbic acid antioxidant system in animals. The Journal of biological chemistry. 1994;269(13):9397-400.
168. Hall AG. Review: The role of glutathione in the regulation of apoptosis. European journal of clinical investigation. 1999;29(3):238-45.
169. Arrick BA, Nathan CF. Glutathione metabolism as a determinant of therapeutic efficacy: a review. Cancer research. 1984;44(10):4224-32.

APPENDIX

APPENDIX 1 Blood chemistry parameters of splenectomized β-thalassemia/Hb E patients

Name	Sex	Age	Blood chemistry parameters							
			BUN mg/dl	Creatinine mg/dl	Uric acid mg/dl	Cholesterol mg/dl	Triglyceride mg/dl	HDL-CHOL mg/dl	Direct LDL-CHOL mg/dl	Total protein g/dl
NPJ	M	42	13.9	0.5	8.5	143	76	30	99	8.6
APR	M	25	13.2	0.5	4.1	120	122	34	74	8.5
NPN	M	22	14.0	0.6	10.2	68	141	19	33	9.4
PJJ	M	30	13.8	0.4	4.1	116	84	38	41	8.0
GRJ	M	20	15.0	0.6	5.9	97	69	39	32	8.1
TYT	M	28	14.9	0.5	6.6	107	98	36	41	7.9
Mean ± SEM		(n=6)	14.13 ± 0.28	0.52 ± 0.03	6.57 ± 0.99	108.50 ± 10.25	98.33 ± 11.47	32.67 ± 3.03	53.33 ± 11.08	8.42 ± 0.23
VPK	F	21	10.3	0.4	5.5	125	102	25	39	7.9
PLP	F	17	10.0	0.3	4.0	95	212	22	54	7.5
CTM	F	30	8.0	0.4	5.7	96	127	31	55	8.8
APT	F	44	13.0	1.6	8.5	193	136	41	133	7.5
Mean ± SEM		(n=4)	10.33 ± 1.03	0.68 ± 0.31	5.93 ± 0.94	127.25 ± 22.99	144.25 ± 23.70	29.75 ± 4.19	70.25 ± 21.23	7.93 ± 0.31

APPENDIX 1 Blood chemistry parameters of splenectomized β-thalassemia/Hb E patients (Continued)

Name	Sex	Age	Blood chemistry parameters							
			Albumin	Globulin	Total Bilirubin	Direct Bilirubin	AST (SGOT)	ALT (SGPT)	Alkaline (ALP)	GGT
			g/dl	g/dl	mg/dl	mg/dl	U/L	U/L	U/L	U/L
NPJ	M	42	5.0	3.6	5.5	0.7	105	88	97	43
APR	M	25	4.6	3.9	3.1	0.5	56	56	96	48
NPN	M	22	4.3	5.1	7.8	1.4	50	25	209	56
PJJ	M	30	4.4	3.6	5.0	0.6	110	85	158	58
GRJ	M	20	5.0	3.1	3.1	0.4	54	39	119	56
TYT	M	28	4.3	3.6	4.4	0.5	35	23	89	38
Mean ± SEM		(n=6)	4.60 ± 0.13	3.82 ± 0.28	4.82 ± 0.72	0.68 ± 0.15	68.33 ± 12.76	52.67 ± 11.74	128.00 ± 19.19	49.83 ± 3.33
VPK	F	21	4.0	3.9	4.1	0.6	85	59	135	45
PLP	F	17	4.8	2.7	5.1	1.0	140	127	181	116
CTM	F	30	4.4	4.4	3.9	0.5	101	84	89	81
APT	F	44	3.8	3.7	1.4	0.3	64	72	174	36
Mean ± SEM		(n=4)	4.25 ± 0.22	3.68 ± 0.36	3.63 ± 0.79	0.60 ± 0.15	97.50 ± 16.06	85.50 ± 14.75	144.75 ± 21.16	69.50 ± 18.30

APPENDIX 2 Blood chemistry parameters of non-splenectomized β-thalassemia/Hb E patients

Name	Sex	Age	Blood chemistry parameters							
			BUN mg/dl	Creatinine mg/dl	Uric acid mg/dl	Cholesterol mg/dl	Triglyceride mg/dl	HDL-CHOL mg/dl	Direct LDL-CHOL mg/dl	Total protein g/dl
CNB	M	45	37.6	1.1	9.2	146	77	44	83	5.9
SRP	M	52	14.6	0.3	6.7	62	77	20	31	8.9
VSB	M	16	11.4	0.4	8.1	125	85	26	46	8.3
TSC	M	19	9.0	0.3	9.2	72	89	25	38	7.7
DNN	M	18	12.0	0.4	8.5	69	100	25	39	7.5
SPS	M	41	17.0	0.8	10.3	120	103	24	81	7.8
TNK	M	39	15.0	0.8	8.1	84	100	22	30	8.0
SYU	M	27	15.2	0.8	6.7	105	95	22	35	8.3
Mean ± SEM		(n=8)	16.48 ± 3.15	0.61 ± 0.11	8.35 ± 0.44	97.88 ± 10.84	90.75 ± 3.67	26.00 ± 2.67	47.88 ± 7.65	7.80 ± 0.31
NPJ	F	22	10.3	0.2	7.8	73	137	25	40	7.3
CLK	F	31	16.6	0.4	5.6	104	65	39	55	8.2
BYR	F	54	11.4	0.4	8.1	125	85	26	46	8.3
GST	F	30	15.0	0.5	7.3	101	117	28	60	8.1
YPN	F	21	14.0	0.6	7.3	102	115	27	59	8.0
JTJ	F	35	13.0	0.5	7.2	98	120	25	61	7.9
BSP	F	49	13.0	0.4	5.9	74	115	23	32	8.1
PGS	F	24	14.0	0.9	9.8	76	147	26	34	8.0
APP	F	24	10.2	0.6	5.3	65	57	21	38	8.3
PTN	F	28	13.1	0.4	4.3	87	101	28	31	8.3
VTY	F	27	16.0	0.8	8	132	128	29	29	7.9
VPJ	F	34	18.3	0.8	9.1	86	55	21	31	7.3
Mean ± SEM		(n=12)	13.74 ± 0.71	0.54 ± 0.06	7.14 ± 0.46	93.58 ± 6.00	103.5 ± 8.99	26.50 ± 1.36	43.00 ± 3.63	7.98 ± 0.10

APPENDIX 2 Blood chemistry parameters of non-splenectomized β-thalassemia/Hb E patients (Continued)

Name	Sex	Age	Blood chemistry parameters							
			Albumin	Globulin	Total Bilirubin	Direct Bilirubin	AST (SGOT)	ALT (SGPT)	Alkaline (ALP)	GGT
			g/dl	g/dl	mg/dl	mg/dl	U/L	U/L	U/L	U/L
CNB	M	45	3.6	2.3	2.7	0.5	29	9	103	25
SRP	M	52	4.2	4.7	5.2	0.7	74	42	70	33
VSB	M	16	4.4	3.9	4.5	0.4	54	39	88	41
TSC	M	19	4.9	2.8	4.0	0.3	25	15	214	16
DNN	M	18	5.3	2.2	4.7	0.3	25	7	141	17
SPS	M	41	4.8	3.0	5.6	0.8	41	41	69	50
TNK	M	39	4.2	3.8	3.5	0.6	39	50	124	34
SYU	M	27	5.0	3.3	3.9	0.4	73	53	161	78
Mean ± SEM		(n=8)	4.55 ± 0.19	3.25 ± 0.30	4.36 ± 0.33	0.50 ± 0.07	45.00 ± 7.09	32.00 ± 6.60	121.25 ± 17.61	36.75 ± 7.17
NPJ	F	22	4.4	2.9	2.8	0.3	9	8	68	18
CLK	F	31	4.8	3.4	1.9	0.4	29	51	71	27
BYR	F	54	4.4	3.9	4.5	0.4	54	39	88	41
GST	F	30	4.6	3.5	4.0	0.6	57	46	118	38
YPN	F	21	4.6	3.4	4.1	0.6	59	45	120	39
JTJ	F	35	4.4	3.5	3.9	0.5	61	47	114	42
BSP	F	49	4.7	3.4	3.4	0.6	35	7	68	10
PGS	F	24	5.3	2.7	2.6	0.7	13	5	47	7
APP	F	24	4.8	3.5	5.1	0.6	25	19	65	41
PTN	F	28	5.1	3.2	6.1	0.7	71	54	165	55
VTY	F	27	4.8	3.1	4.3	0.5	34	20	98	43
VPJ	F	34	4.4	2.9	2.8	0.3	44	31	85	22
Mean ± SEM		(n=12)	4.69 ± 0.08	3.28 ± 0.10	3.79 ± 0.34	0.52 ± 0.04	40.92 ± 5.74	31.00 ± 5.31	92.5 ± 9.42	31.92 ± 4.29

APPENDIX 3 Baseline blood chemistry parameters of β-thalassemia/Hb E patients

Parameters		Reference Range	β-thalassemia/Hb E patients		
			Splenectomized	non Splenectomized	Total
			n=10	n=20	n=30
BUN	(mg/dl)	7.0 – 20.0	12.61 ± 0.74	14.84 ± 1.32	14.09 ± 0.92
Creatinine	(mg/dl)	2.4 – 7.0	0.58 ± 0.12	0.57 ± 0.05	0.57 ± 0.05
Uric acid	(mg/dl)	100 – 200	6.31 ± 0.68	7.63 ± 0.35	7.19 ± 0.34
Cholesterol	(mg/dl)	50 – 200	116.00 ± 10.72	95.30 ± 5.48	102.20 ± 5.32
Triglyceride	(mg/dl)	35 – 100	116.70 ± 13.23	98.40 ± 5.67	104.50 ± 5.89
HDL-CHOL	(mg/dl)	70 – 160	31.50 ± 2.37	26.30 ± 1.30	28.03 ± 1.24
Direct LDL-CHOL	(mg/dl)	6.6 – 8.7	60.10 ± 10.43	44.95 ± 3.68	50.00 ± 4.35
Total protein	(g/dl)	3.5 – 5.5	8.22 ± 0.19	7.91 ± 0.14	8.01 ± 0.11
Albumin	(g/dl)	1.5 – 3.5	4.46 ± 0.13	4.64 ± 0.09	4.58 ± 0.07
Globulin	(g/dl)	0.3 – 1.2	3.76 ± 0.21	3.27 ± 0.13	3.43 ± 0.12
Total Bilirubin	(mg/dl)	0 – 0.5	4.34 ± 0.54	3.98 ± 0.24	4.10 ± 0.24
Direct Bilirubin	(mg/dl)	0 – 37	0.65 ± 0.10	0.51 ± 0.04	0.56 ± 0.04
AST (SGOT)	(U/L)	0 – 40	80.00 ± 10.55	42.55 ± 4.37	55.03 ± 5.53
ALT (SGPT)	(U/L)	39 – 117	65.80 ± 10.18	31.40 ± 4.03	42.87 ± 5.18
Alkaline (ALP)	(U/L)	7 – 50	134.70 ± 13.78	103.85 ± 9.34	114.13 ± 8.06
GGT	(U/L)		57.70 ± 7.66	33.85 ± 3.77	41.80 ± 4.08

*Values were given as mean ± SEM

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