EFFECT OF RICEBERRY BRAN OIL ON ANTIOXIDANTS AND OXIDATIVE STRESS STATUS OF NORMAL AND BETA-THALASSEMIC MICE

KANIN TEERATANTIKANON

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…….
Miss Kanin Teeratantikanon
Candidate

……..…
Assoc. Prof. Prapaisri P. Sirichakwal,
Ph.D. (Nutritional Biochemistry and Metabolism)
Major-advisor

…….……
Prof. Suthat Fucharoen,
M.D.
Co-advisor

……..
Lect. Aikkarach Kettawan,
Ph.D. (Pharmaceutical Science)
Co-advisor

……..
Asst. Prof. Noppawan Phumala Morales,
Ph.D. (Pharmaceutical Science)
Co-advisor

…….
Prof. Banchong Mahaisavariya,
M.D., Dip Thai Board of Orthopedics
Dean
Faculty of Graduate Studies
Mahidol University

……..
Prof. Surat Komindr,
M.D., F.R.C.PCT
Program Director
Doctor of Philosophy Program in Nutrition
Faculty of Medicine, Ramathibodi Hospital and Institute of Nutrition
Mahidol University

Thesis
entitled

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on
August 26, 2010

……..
Miss Kanin Teeratantikanon
Candidate

……..……	……..…
Prof. Suthat Fucharoen, M.D. Member	Prof. Sakorn Dhanamitta, M.D. D.Sc. (Medicine) Chair
……	……
Lect.Aikkarach Kettawan, Ph.D. (Pharmaceutical Science) Member	Assoc. Prof. Prapaisri P. Sirichakwal, Ph.D. (Nutritional Biochemistry and Metabolism) Member
……	……..
Asst. Prof. Noppawan Phumala Morales, Ph.D. (Pharmaceutical Science) Member	Assoc. Prof. Visith Chavasit, Ph.D. (Food Science) Director Institute of Nutrition, Mahidol University
……	……..
Prof. Banchong Mahaisavariya, M.D., Dip Thai Board of Orthopedics Dean Faculty of Graduate Studies Mahidol University	Prof. Rajata Rajatanavin, M.D., F.A.C.E. Dean Faculty of Medicine, Ramathibodi Hospital Mahidol University

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KANIN TEERATANTIKANON 4936964 RANU/M

M.Sc. (NUTRITION)

THESIS ADVISORY COMMITTEE : PRAPAISRI P SIRICHAKWAL, Ph.D., SUTHAT FUCHAROEN, M.D., AIKKARACH KETTAWAN, Ph.D., NOPPAWAN PHUMALA MORALES, Ph.D.

ABSTRACT

β-thalassemia is a group of genetic disorders resulting from different mutations in the globin gene complex which lead to imbalance in β-globin chain synthesis. Unmatched α-globin chains are less stable and induce oxidation. In addition, ineffective erythropoiesis and iron overload lead to rise of free radicals. Riceberry bran oil, extracted from bran of Riceberry rice, a variety developed by crossed breeding of Khao Dawk Mali 105 and Chao Hom Nil, contains a variety of antioxidative agents. This study was conducted to evaluate the effect of Riceberry bran oil on antioxidants and oxidative stress status of normal and β-thalassemic mice with pathophysiologic changes similar to β-thalassemia intermedia patients. Control thalassemic mice showed higher oxidative stress as indicated by increased reactive oxygen species (ROS) in red blood cells and decreased lipid fluidity in the area of the hydrophobic region of the red cell membrane compared to the control normal mice, while the level of antioxidant CoQ_{10} was lower. In addition, lower total cholesterol but higher hepatic iron content and higher heart and spleen weights were detected in thalassemic mice. Low dose (17 mg/20g body wt) and high dose (36 mg/20g body wt) of Riceberry bran oil were given in normal mice and thalassemic mice for 2 months; serum antioxidants and oxidative stress status were measured compared to normal mice. Two doses of Riceberry bran oil increased CoQ_{10}, lipid fluidity in the area of the hydrophobic region of the red cell membrane and decreased triglyceride. They tended to increase vitamin E in thalassemic mice and decrease lipid peroxidation in normal and thalassemic mice. However, no change in superoxide dismutase, free cholesterol or cholesteryl esters were observed. No adverse effect of the Riceberry bran oil was found on liver and renal profiles, hepatic malondialdehyde, hepatic iron content, red cell morphology or organs in any group.

KEYWORDS: RICEBERRY BRAN OIL / BETA-THALASSEMIC MICE / ANTIOXIDANT/ OXIDATIVE STRESS STATUS

130 pages

การศึกษาผลการให้น้ำมันรำข้าวไรซ์เบอร์รีต่อภาวะสารต้านอนุมูลอิสระและออกซิเดทีฟสเตรสในหนูปกติและหนูธาลัสซีเมียชนิดเบต้า

EFFECT OF RICEBERRY BRAN OIL ON ANTIOXIDANTS AND OXIDATIVE STRESS STATUS OF NORMAL AND BETA-THALASSEMIC MICE

กนิน ธีระตันติกานนท์ 4936964 RANU/M

วท.ม. (โภชนศาสตร์)

คณะกรรมการที่ปรึกษาวิทยานิพนธ์ : ประไพศรี ศิริจักรวาล, Ph.D., สุทัศน์ ฟู่เจริญ, M.D., เอกราช เกตวัลห์, Ph.D., นพวรรณ ภู่มาลา มอราเลส, Ph.D.

บทคัดย่อ

เบต้า-ธาลัสซีเมียเป็นกลุ่มของความผิดปกติทางพันธุกรรมเป็นผลมาจากการเกิดมิวเตชั่นหลายแบบในโกลบินยีน นำไปสู่ความไม่สมดุลในการสังเคราะห์สายเบต้า-โกลบิน สายแอลฟ่า-โกลบินที่ไม่มีคู่ จึงไม่เสถียรทำให้เกิดออกซิเดชั่น นอกจากนี้ การสร้างเม็ดเลือดแดงที่ไม่มีประสิทธิภาพและภาวะเหล็กเกินนำไปสู่ การเพิ่มขึ้นของอนุมูลอิสระ น้ำมันรำข้าวไรซ์เบอร์รีสกัดจากรำข้าวของข้าวไรซ์เบอร์รี ซึ่งเป็นพันธุ์ข้าวที่พัฒนาโดยการผสมจากข้าวขาวดอกมะลิ 105 และข้าวเจ้าหอมนิล อุดมไปด้วยความหลากหลายของสารต้านอนุมูลอิสระ การศึกษานี้จึงดำเนินการ เพื่อประเมินผลการให้น้ำมันรำข้าวต่อภาวะสารต้านอนุมูลอิสระและออกซิเดทีฟสเตรสในหนูปกติและหนูธาลัสซีเมียชนิดเบต้าซึ่งมีการเปลี่ยนแปลงพยาธิสรีรวิทยาคล้ายคลึงกับที่เกิดในผู้ป่วยเบต้า-ธาลัสซีเมียชนิด intermedia โดยพบว่า หนูธาลัสซีเมียกลุ่มควบคุมมีภาวะออกซิเดทีฟสเตรสสูงกว่าหนูปกติกลุ่มควบคุม แสดงให้เห็นจากการเพิ่มขึ้นของ reactive oxygen species (ROS) ในเม็ดเลือดแดงและการเคลื่อนไหวลดลงของไขมันระดับโมเลกุลในบริเวณ hydrophobic ของผนังของเม็ดเลือดแดง ในขณะที่ระดับของสารต้านอนุมูลอิสระ CoQ_{10} มีค่าต่ำกว่า นอกจากนี้ หนูธาลัสซีเมีย ยังมีระดับโคเลสเตอรอลในเลือด ที่ต่ำกว่า หนูปกติ แต่ปริมาณเหล็กสะสมที่ตับมากกว่าและน้ำหนักหัวใจและม้ามที่ สูงกว่า ภายหลังที่หนูธาลัสซีเมียได้รับน้ำมันรำข้าวไรซ์เบอ รีในปริมาณต่ำ (17 มก./20 กรัมของน้ำหนักตัว) และปริมาณสูง (36 มก./20 กรัมของน้ำหนักตัว) เป็นเวลา 2 เดือน มีการวัดภาวะสารต้านอนุมูลอิสระและออกซิเดทีฟสเตรส เปรียบเทียบกับหนูปกติ พบว่า น้ำมันรำข้าวไรซ์เบอร์ รีทั้ง 2 ระดับเพิ่ม CoQ_{10} และการเคลื่อนไหวของไขมันระดับโมเลกุลในบริเวณ hydrophobic ของผนังของเม็ดเลือดแดงและ ลดไตรกลีเซอไรด์ มีแนวโน้มในการเพิ่มวิตามินอีในหนูธาลัสซีเมียและลดการเกิดออกซิเดชั่นของไขมันทั้งในหนูปกติและหนูธาลัสซีเมีย อย่างไรก็ตามไม่มีการเปลี่ยนแปลงใน superoxide dismutase, free cholesterol และ cholesteryl esters น้ำมันรำข้าวไรซ์เบอร์รี ไม่มีผลต่อตัวชี้วัดการทำงานของตับและไต , การเกิดออกซิเดชั่นของไขมันที่ตับ, ปริมาณเหล็กสะสมที่ตับ, ลักษณะของเม็ดเลือดแดงและอวัยวะในทุกกลุ่ม

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LIST OF ABBREVIATIONS

ALK	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspatate aminotransferase
BKO	Beta-globin gene knocked-out
CA	Cholesteryl arachidonate
CAT	Catalase
CEs	Cholesteryl esters
CL	Cholesteryl linoleate
CoQ_{10}	Coenzyme Q10
CuZnSOD	Cu/Zn containing SOD
5-DS	5-doxyl stearic acid
16-DS	16-doxyl stearic acid
FC	Free cholesterol
g	gram
GSH-Px	Glutathione peroxidase
Hb	Hemoglobin
HDL-C	High density lipoprotein cholesterol
HMG CoA-R	3-hydroxy-3-methyl-glutaryl-CoA reductase
HPLC-ECD	High performance liquid chromatography with electrochemical detector
HPRT	Hypoxanthine phosphoribosyl transferase
LDL-C	Low density lipoprotein cholesterol
MCH	Mean Corpuscular Hemoglobin
MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean Corpuscular Volumn
MDA	Malondialdehyde
MnSOD	Manganese containing SOD
8-OHdG	8-hydroxy-2′-deoxyguanosine

LIST OF ABBREVIATIONS (cont.)

µg	microgram
mg	milligram
ml	milliliter
mol	mole
MUFA	Monounsaturated fatty acid
NADP	Nicotinamide adenosine dinucleotide-2′phosphate
NADPH	Reduced nicotinamide adenosine dinucleotide-2‘ phosphate
NDEA	N-nitrosodiethylamine
ng	nanogram
NIBT	Non-transferrin bound iron
ORAC	Oxygen radical absorbance capacity
PL	Phospholipids
PPP	Pentose Phosphate Pathway
PUFAs	Polyunsaturated fatty acids
RDW	Red cell distribution widths
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SFA	Saturated fatty acid
STZ	Streptozotocin
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TC	Total cholesterol
tk	Thymidine kinase
TG	Triglyceride

CHAPTER I
INTRODUCTION

Thalassemia is a diverse group of genetic disorder of hemoglobin synthesis. Normally, hemoglobin consists of four globin chains (two chains of alpha-globin and two chains of beta-globin). Thalassemia defects range from decreased synthesis to the absence of the affected globin chains leading to the accumulation of the excess unaffected globin chain which aggregate and precipitate within the red blood cell precursors and their progeny. This leads to toxic to the red blood cells and results in ineffective and reduced red cell life span. These imbalance globin chain synthesis defective hemoglobin production characterized by small hemoglobinized red cells, microcytosis, hypochromia with low Mean Corpuscular Volume (MCV) and Mean Corpuscular Hemoglobin (MCH) value (1). Thalassemia can be divided into two major groups, α- and β-thalassemia. The frequencies are high in Southeast Asia (SE) including Thailand (2).

Oxidative stress of β-thalassemic red blood cells

The oxidative stress is the set of intracellular or extracellular conditions that leads to the chemical or metabolic generation of reactive oxygen species (ROS), such as superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), hydrogen peroxide (H_2O_2) or related species. It can result from diminished antioxidants and / or increased production of ROS. The oxidative stress results in adaptation and cell injury. β-thalassemic red blood cells are susceptible to oxidative stress. Because of the aggregation of the excess of α-chains can cause oxidation furthermore; these globin chains can synthesize more methemoglobin. In case of thalassemia has low hemoglobin so they could not eliminate oxidation that occurs in the cell. Free α-chains tend to be oxidized, first to methemoglobin and later irreversible hemichromes. In thalassemic erythrocyte, the unstable hemoglobin and isolated hemoglobin chains

are associated with increase rates of methemoglobin formation and then increased releases of superoxide (O_2^-). In addition, ineffective erythropoiesis and iron overload lead to reduction of antioxidant by premature destruction of the red blood cells together with enhanced intestinal absorption is the major contributing sources of the surplus iron in thalassemia blood. Level of serum iron is elevated as transferrin becomes fully saturated and the appearance of non-transferrin bound iron (NTBI). The later circulating pool of iron determines the toxicity of iron overload or the varying degree of oxidative stress in thalassemia. Lipid peroxidation shows a good correction figure to the body iron status. Iron catalyzed formation of oxygen-derived reactive oxygen species (ROS) by the well known "Fenton-Harber Weiss" reaction is eminent in thalassemia consequently the depletion of antioxidants (3).

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

$$H_2O_2 + OH\bullet \longrightarrow H_2O + O_2^- + H^+$$

$$H_2O_2 + O_2\text{-} \longrightarrow O_2 + OH^- + OH\bullet$$

The studies found that, the lipid peroxidation and proteins hydrolysate at the red blood cell membrane of thalassemia patients because these red blood cells are activated oxygen than usual (4-5).

The summary factors concluded that thalassemic erythrocytes are susceptible to oxidation and destruction leading to a shortened erythrocytes survival, hepatosplenomegaly and anemia that is similar to the iron deficiency, however, iron supplementation is not beneficial because it increases oxidation. Therefore, if there are a method to increases the functionality of antioxidant, make a better oxidative stress and strengthening of red blood cells, it will help to improve patients for a better quality of life.

Antioxidants are potentially protective to guard against oxidative hemolysis, protecting the red blood cells and prolonging red blood cell lifespan in thalassemia patients (6).

Antioxidant is well known as the first line of defense against lipid peroxide both *in vivo* and *in vitro* is vitamin E (7-8). Previous study found that the thalassemia patients had significantly lower plasma vitamin E status than normal children (9). After supplemented with 200 mg of oral vitamin E for 4-8 weeks, their plasma vitamin E increased and H_2O_2 hemolysis decreased to normal values (10).

Many studies founded that rice bran oil is a good source of vitamin E and a variety of antioxidative agents such as β-carotene, gamma-oryzanol, CoQ_{10} and polyphenol (11-16). This is appropriate for supplement in oxidative stress risk person such as to reduce the risk of unusual oxidation in thalassemia patient.

Chanphrom *et al.* and Kongkachuichai *et al*. determined antioxidant activities and antioxidant contents of purple non-glutinous brown rice, rice bran and rice bran oil samples from Rice Science Research Center, Kasetsart University, Kamphangsaen, Nakhon-Pathom, Thailand. This studies found that rice bran and rice bran oil # 1000-11-2-26 (Riceberry) had antioxidant activities using oxygen radical absorbance capacity (H-ORAC) and antioxidant contents such as vitamin E, β-carotene, lutein, polyphenol, anthocyanidin, gamma-oryzanol and CoQ_{10} (17-18).

Previously, no study using rice bran oil on antioxidant and oxidative stress in thalassemia patients has been demonstrated. Thus, this research study performed in thalassemic mice prior to thalassemia patients. β-thalassemic mice from Thalassemia Research Center, Institute of Molecular Biosciences, Mahidol University were used due to the similarity of pathophysiologic changes, clinical and erythrocyte features of thalassemic mice and human β-thalassemia intermedia. In addition, Riceberry bran oil was supplied from Rice Science Research Center, Kasetsart University, Kamphangsaen, Nakhon-Pathom, Thailand. If this study successfully as expected, the trial study in thalassemia patients need to be further explored in order to confirm the beneficial effect of this natural product for a treatment of oxidative stress which will lead to improve the quality of life of the patients.

Objectives

General Objective

To study the effect of rice bran oil on antioxidants and oxidative stress status of normal and β-thalassemic mice.

Specific Objectives

To study the effect different doses of Riceberry bran oil on normal mice (control and high dose) and β-thalassemic mice (control, low dose and high dose).

1. **Oxidative stress status and antioxidants**: Reactive Oxygen Species (ROS), Lipid fluidity, Malondialdehyde (MDA), Superoxide dismutase (SOD), CoQ_{10} and Vitamin E
2. **Biochemical assessment**: Free cholesterol (FC), Cholesteryl esters (CEs), Total cholesterol (TC), Triglyceride (TG), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALK), Total protein, albumin, globulin, total bilirubin and uric acid
3. **Hematological assessment**: Red blood cell morphology
4. **Pathological assessment**: Hepatic malondialdehyde, Hepatic iron content and histopathologic studies of livers

CHAPTER II
LITERATURE REVIEW

2.1 Thalassemia

Thalassemia can be divided into two main a hereditary groups disorder of hemoglobin synthesis

2.1.1 α-thalassemia

α-thalassemias are caused by a decrease in the production of α-globin chains by the deletion or mutation of one or both of α-globin genes in the α-globin locus located on chromosome 16. There are two types of α-thalassemia: α-thalassemia 1 or α° thalassemia, which caused by the loss of both linked α-globin genes leads to no α-globin chain synthesis (Hb Bart's hydrops fetalis). α-thalassemia 2 or α^+ thalassemia, which caused by the loss of only α-globin gene and leads to reduced synthesis of α-globin chains (Hb H disease).

2.1.2 β-thalassemia

β-thalassemias are characterized by reduction or absence in the β globin chain synthesis. In contrast, to the α-thalassemias, most β-thalassemias are caused by mutation affecting gene regulation or expression rather than gene deletion. The β-thalassemias are caused by more than 200 mutations on chromosome 11 in the functionally important regions of the β-globin gene which result in a deficit of the β-globin chain production (19). The β-thalassemias are subdivided into β°-thalassemia, which there is no β-globin chain production, and β^+-thalassemia, which there is a reduced of the β-globin chain production.

2.2 Pathophysiology of β-thalassemia

The results from mutation at the β-globin gene lead to either an absence of the β-globin chain production (β° thalassemia) or a variable reduction in the output (β^+

thalassemia). This leads to imbalanced globin synthesis and an excess of α-chains that produce the large size of the free α-chain pool. Excess α-chains precipitate in red cell membrane and red cell precursor in bone marrow in their progeny in peripheral blood leading to defective erythroid precursor maturation, ineffective erythropoiesis and a shortened red cell survival. The resulting anemia causes an intense proliferative drive in the ineffective bone marrow which leads to its expansion. This results cause skeletal deformities, a variety of growth and metabolic abnormalities. The hyperplasia of the bone marrow leads to increased iron absorption and iron loading resulting in a progressive iron deposition in the tissues, organ failure and, if the iron is not removed, may cause life-threatening.

2.3 Clinical classification of β-thalassemia

The β-thalassemia can be classified into three groups

2.3.1 Thalassemia minor

Thalassemia minor is an asymptomatic illness and usually associated with the inheritance of a single β-thalassemia allele, whether β^+ or β°, and characterized by a mild anemia with hypochromic microcytic red blood cells.

2.3.2 Thalassemia intermedia

Signs and symptoms of this group are comparable to those of thalassemia major but of a lesser severity of disease. Approximate 75 % of patients with β-thalassemia intermedia are homozygous or compound heterozygous and that 50 % of these patients had inherited one or two mild β-thalassemia gene (20). Thalassemia intermedia has a diversity disorders, from almost complete health, not require blood transfusion to a condition characterized by severe growth retardation and skeletal deformity requiring transfusion therapy. For example, the most common severe thalassemia intermedia in South East Asia, the compound heterozygous state Hb E/ β°-thalassemia has hemoglobin value varying from 2 to 12 g/dl (21). However, most patients have hemoglobin value between 6 and 8 g/dl with hepatosplenomegaly, grow and develop reasonably well and reach adult life. β-Thalassemia intermedia such as β-thalassemia/ Hb E disease and β-thalassemia/ Hereditary Peristence of Fetal Hemoglobin.

2.3.3 Thalassemia major

This group is the most severe form of thalassemia disease. Patients have all signs and symptoms development associated with severe anemia, growth retardation, tower skull, hepatosplenomegaly and thalassemia faces. This disease is usually fatal early in life. Chronic blood transfusions are needed in these patients. While transfusions are lifesaving for these patients but they result in iron overload. β-thalassemia major usually results from homozygous state for either β^+ or β° thalassemia, or from the compound heterozygous state for either β^+ or β° thalassemia.

2.4 Thalassemic mouse models

The mouse β-globin gene cluster located on chromosome 7 contains 4 functional β-like globin genes: βh1, an embryonic globin gene, $\varepsilon\gamma^2$, a late embryonic globin gene and two adult β-globin genes, β^{major} or *b1* and β^{minor} or *b2* (Figure 2.1) (22).

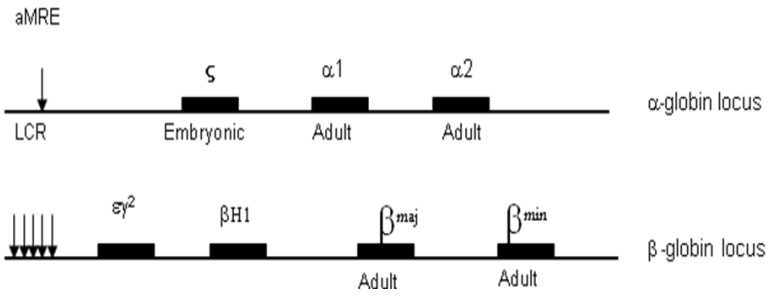


Figure 2.1 Globin locus of normal mice

Mouse embryonic hemoglobins are first expressed at 9.5 days of gestation in the yolk sac and later in liver of fetus. The switch from predominantly embryonic to predominantly adult hemoglobins is completed between days 14 and 15 of gestation in the normal fetus (23-24). The β^{major} and β^{minor} are then expressed in fetal liver, spleen and finally in bone marrow during adult life. The β^{major} gene is reported to be responsible for about 80 percent and the β^{minor} gene for about 20 percent of adult β-globin production (25).

The β°-thalassemic mouse models were generated using C57BL/6J strain ($m\alpha^{+\,+}/m\beta^{+\,+}$) as follows.

2.4.1 Heterozygous β-globin gene knock-out mice (BKO, $m\alpha^{++}/m\beta^{+-}$)

Heterozygous β-globin gene knock-out mice (BKO, $m\alpha^{++}/m\beta^{+-}$) which carry a complete deletion of adult murine β-globin genes and intact β-globin gene allele. This mouse model are carried a 20 kb deletion encompassing the entire mouse β^{major} and β^{minor} gene was generated by using a plug and socket method and a functional neomycin resistant (*nero*^{R}) gene together with a nonfunctional hypoxanthine phosphoribosyltransferase (HPRT) minigene for double selection (26). Mice heterozygous appear normal, but their hematologic indices show characteristics typical of severe anemia, dramatically decreased hemoglobin, hematocrit, red blood counts, Mean Corpuscular Volumn (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC) as well as dramatically increased reticulocyte counts, serum bilirubin concentrations and red cell distribution widths (RDW). Tissue and organ damage typical of β-thalassemia such as bone deformities and splenomegaly due to increased hemopoiesis are seen as well as iron overload in spleen, liver and kidney (27). The increase of oxidative stress as indicated by the level of reactive oxygen species (ROS) in β-thalassemic mice of this phenotypes (β-knockout) was significantly higher (P<0.0001) than that in the normal mice group (wild type) and the other phenotypes of β-thalassemic mice such as Hb E-trangenic heterozygote (HbE-TG) and HbE-double heterozygote (Hb E-DH). In addition, the red blood cells of β-knockout had a shortened life span (the mean survival half life was significantly decreased ($T_{1/2}$ = 12.3±1.39 days) when compared with normal mice (wild type) ($T_{1/2}$ = 12.3±1.39 days)), indicating that high red cell turnover rates and correlated with increased percentage of reticulocyte production (28). The above findings, as well as several other studies, indicated that features of these thalassemic mice are similar to those found in human β-thalassemia intermedia (27-29).

2.4.2 Homozygous β-globin gene knock-out mice

Homozygous β-knocked out mice die within hours after birth rather than later in childhood, unlike humans. This mouse model are carried a 16 kb deletion encompassing the entire mouse β^{major} gene and most of the β^{minor} gene was generated by using a targeting construct composed of the neomycin resistant (*neo*^{R}) and thymidine kinase (*tk*) genes (30).

2.5 Free radical and oxidative stress

2.5.1 Free radical

Free radical is an atom or molecules with one or more unpaired electrons. These free radicals are usually unstable and highly reactive because the unpaired electrons tend to form pairs with other electrons from another molecule or substances. Many of the important free radical involving oxygen can be referred to as reactive oxygen species (ROS). Examples of reactive oxygen species (ROS) are superoxide ($O_2^{\bullet-}$) and hydroxyl radical ($OH^{\bullet}$). Any important radical involving nitrogen can be referred to as reactive nitrogen species (RNS) such as nitric oxide ($NO^{\bullet}$) and peroxynitrite (ONOO). In addition there are some important intermediates which are not free radical but which are highly reactive and may be responsible for some of the biologic effects attributed to free radicals, such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCL) (31) (**Table 2.1**)

Table 2.1 Reactive Species (32)

Radicals	Non-radicals
Reactive oxygen species (ROS)	Hydrogen peroxide (H_2O_2)
Superoxide ($O_2^{-\bullet}$)	Hypocholorous acid (HOCl)
Hydroxyl ($OH^{\bullet}$)	Ozone (O_3)
Hydroperoxyl ($HO_2^{\bullet}$)	Singlet oxygen ($O_2^{\bullet}$)
Peroxyl ($RO_2^{\bullet}$)	Peroxylnitrite ($ONOO^-$)
Alkoxyl ($RO^{\bullet}$)	
Reactive nitrogen species (RNS)	
Nitric oxide ($NO^{\bullet}$)	
Nitrogen dioxide ($NO_2^{\bullet}$)	

2.5.2 Oxidative stress

Oxidative stress has been defined as a disturbance in equilibrium status between oxidizing agent or oxidant and antioxidant in intact cell. Oxidative stress which results in up-regulation of the synthesis of antioxidant/ antioxidant balance. If

the oxidative stress increases in cell, it can damage all type of bimolecular including DNA, protein and lipid. The primary cellular target of the oxidative stress can vary depending on the cell, the type of oxidative stress imposed and severity of stress. In addition, it can damage multiplicity of targets in living cells and the initial damage to one target can then affect other. Diseases cause from oxidative stress such as cancer, diabetes, rheumatoid arthritis, cerebrovascular disease, cataracts and cardiovascular disease (32-33).

2.5.2.1 Malondialdehyde (MDA)

Malondialdehyde is one of the most frequently which used as indicators of lipid peroxidation of polyunsaturated fatty acids (PUFAs). Lipid peroxidation is to represent a primary event in oxidative cellular damage (34). The process of lipid peroxidation has three phases such as initiation, propagation and termination. In the initiation phase (reaction I), carbon-centered lipid radical (R•) can be produced by proton abstraction from a polyunsaturated fatty acid (RH) when free radical initiator (I^*) is present. During the propagation phase, the lipid radical reacts readily with molecular oxygen to form a peroxyl radical (ROO•) (reaction II). The peroxyl radical formed can react with another polyunsaturated fatty acid to form a hydroperoxide (ROOH) and a new carbon-centered radical (reaction III). The propagative process can continue until all polyunsaturated fat is consumed or the chain reaction is broken (termination phase). Free radicals can be scavenged and chain reaction terminated by self-quenching (reaction IV) or by the action of antioxidant (AH) (reaction V) which generates A• of the free radical process inhibitors or antioxidants that are known (35).

(Reaction I) $RH \xrightarrow{I^*} R\bullet$

(Reaction II) $R\bullet + O_2 \longrightarrow ROO$

(Reaction III) $ROO\bullet + R'H \longrightarrow ROOH + R'\bullet$

(Reaction IV) $R\bullet \text{ (or } ROO\bullet) + R'\bullet \longrightarrow R\text{-}R' \text{ (or } ROOR')$

(Reaction V) $R\bullet \text{ (or } ROO\bullet) + AH \longrightarrow RH \text{ (or } ROOH) + A\bullet$

2.5.2.2 Membrane fluidity

Biology membrane is a dynamic molecular assembly characterized by the coexistence of structures with highly restricted mobility and components having great rotational freedom. These membrane lipids and proteins comprising domains of highly restricted mobility appear to exist on a micrometer scale in a number of cell types (36-37). Despite this heterogeneity, membrane fluidity is still considered as a bulk, uniform property of the lipid phase that is governed by a complex pattern of the components' mobility. Individual lipid molecules can display diffusion of three different types: lateral, rotational and transversal (38). Lateral diffusion of lipids in biological membranes refers to the two-dimensional translocation of the molecules in the plane of the membrane. Rotational diffusion of lipid molecules is restricted to the plane of biological membranes, whereas transverse diffusion is the out-of-plane rotation or redistribution of lipid molecules between the two leaflets of the bilayer. Transverse diffusion of flip-flop motion is very low in lipid bilayers, and specific enzymes are required to mediate the process. There are two major components of membrane fluidity. The first component is the order parameter (S), also called the structure, static, or range component of membrane fluidity. This is a measure of angular range of rotational motion, with more tightly packed chains resulting in a more ordered or less fluid bilayer. The second component of membrane fluidity is microviscosity and is the dynamic component of membrane fluidity. This component measures the rate of rotation motion and is more accurate reflection of membrane microviscosity. There are many physical and chemical factors that regulate the fluidity properties of biological membranes, including temperature pressure, membrane potential, fatty acid composition, protein incorporation, and Ca^{2+} concentration. For example, calcium influences the structure of membrane containing acidic phospholipid by nonspecifically cross-linking the negative charges. Consequently, increasing the calcium concentration in systems induced structural rearrangements and a decrease in membrane fluidity (39). Similarly, changes in microfluidity and lateral diffusion fluidity were exhibited when polyunsaturated fatty acid oxidized (40). Fluidity is an important property of membranes because of its role in various cellular functions. Activities of integral membrane-bound enzymes such as Na^+, K^+-ATPase, can be regulated to some extent by changes in the lipid portions of

biological membranes. In turn, changes in enzyme activities tightly connected to ion transport processes could affect translocations of ions.

2.6 Antioxidant defense system

Antioxidant as the compounds that protect biological systems against the potentially harmful effect of processes or reactions that causes extensive oxidation (41). The processes of antioxidant role in one of two ways are chain-breaking or prevention. For the chain-breaking, when a radical steals or releases electron, a second radical is formed. The last one exerts the same action on another molecule and continues until either the free radical formed is stabilized by a chain-breaking antioxidant (vitamin E, carotenoids, etc), or it simply disintegrates into an inactive product. The example of a chain reaction is lipid peroxidation. For the prevention, antioxidant enzyme like superoxide dismutase, catalase and glutathione peroxidase can prevent oxidation by reduce the rate of chain initiation, either by stabilize transition metal radicals such as copper and iron or by scavenge initiating free radicals (42-43). Antioxidant defense systems in cells can be classifies two groups based on their sources as enzymatic antioxidants and non- enzymatic antioxidants.

2.6.1 Enzymatic antioxidants (44-45)

The major of enzymatic antioxidant directly involved in the neutralization of ROS and RNS such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px). The relationship between free reactive oxygen species and enzymatic antioxidants showed in Figure 2.2 (46).

2.6.1.1 Superoxide dismutases (SOD)

Superoxide dismutases are present in all aerobic organisms which provide a defense that is essential for their survival. There are three distinction types of SOD which based on the metal ion in their active sites such as Cu/Zn containing SOD (CuZnSOD), manganese containing SOD (MnSOD) and iron containing SOD (FeSOD). CuZnSOD is the characteristics of eukaryotic cytosols while MnSOD and FeSOD are the characteristics of prokaryotes (47). Superoxide dismutase (SOD) is the first line of defense against free radical and catalyzes the dismutation of superoxide anion radical ($O_2^{\bullet -}$) into hydrogen peroxide (H_2O_2) by

reduction then the oxidant formed (H_2O_2) is transformed into water and oxygen (O_2) by catalase (CAT) or glutathione peroxidase (GSH-Px).

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

2.6.1.2 Catalase (CAT)

Catalase is present in peroxisomes in eukaryotic cells. It directly catalyzes the decomposition of hydrogen peroxide to water and oxygen and finishes the detoxification reaction that started by SOD.

$$2H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2$$

2.6.1.3 Glutathione peroxidase (GSH-Px)

GSH-Px enzyme removes hydrogen peroxide (H_2O_2) by using it to oxidize reduced glutathione (GSH) into oxidized glutathione (GSSG). Glutathione reductase as a flavoprotein enzyme and regenerates GSH from GSSG with reduced nicotinamide adenosine dinucleotide-2′-phosphate (NADPH) as a source of reducing power. In addition, besides hydrogen peroxide, GSH-Px also reduces lipid or nonlipid hydroperoxides while oxidizing glutathione (GSH).

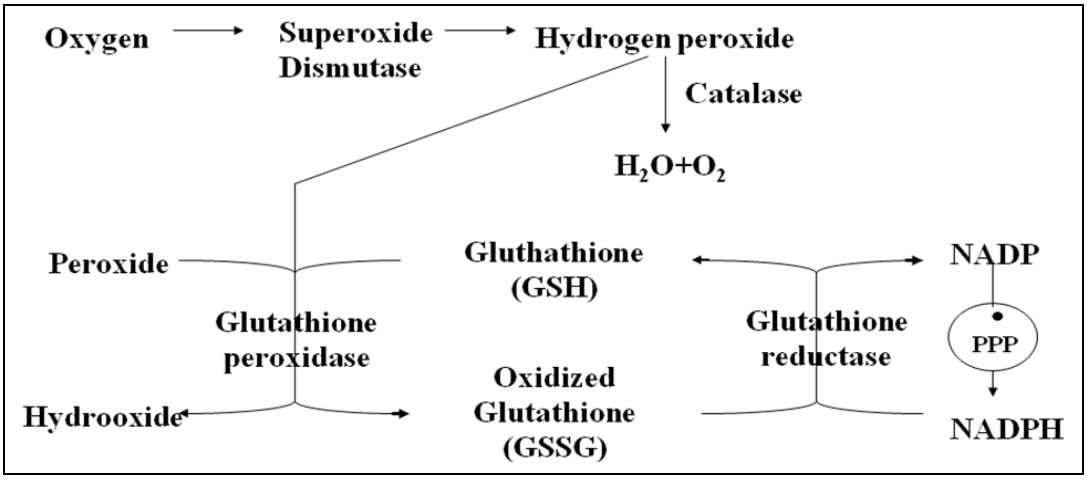


Figure 2.2 Relationship between reactive oxygen species and enzymatic antioxidant (NADP=nicotinamide adenosine dinucleotide-2′ phosphate, NADPH=reduced nicotinamide adenosine dinucleotide-2‘ phosphate, PPP=Pentose Phosphate Pathway)

2.6.2 Non-enzymatic antioxidants

Non-enzymatic antioxidants are also divided into 2 types such as metabolic antioxidants and nutrient antioxidants. Metabolic antioxidants belong to endogenous antioxidants which are produced by metabolism in the body such as L-ariginine, glutathione, lipoid acid, melatonin, coenzyme Q_{10}, uric acid, bilirubin, metal-chelating proteins, transferrin, etc (44). Nutrient antioxidants belong to exogenous antioxidants, which are compounds, cannot be produced in the body and must be provided through foods or supplements such as vitamin E, carotenoids, flavonoids vitamin C, omega-3 and omega-6 fatty acids, trace metals (selenium, manganese, zinc) , etc.

2.7 Rice (48-49)

Rice (*Oryza Sativa L.*) is a cereal grain and the main staple food for two-thirds of the population in the world. Rice cultivation from species *Oryza Sativa L.* which can divide in 3 subspecies such as

- *Japonica* which cultivate in temperature area such as China and Japan
- *Javanica* which cultivate in only Indonesia
- *Indica* which cultivate in temperature area such as Thailand, The Philippines and India

2.7.1 Rice grain structure (50-51)

Rice grain structure consist of following (figure 2.3)

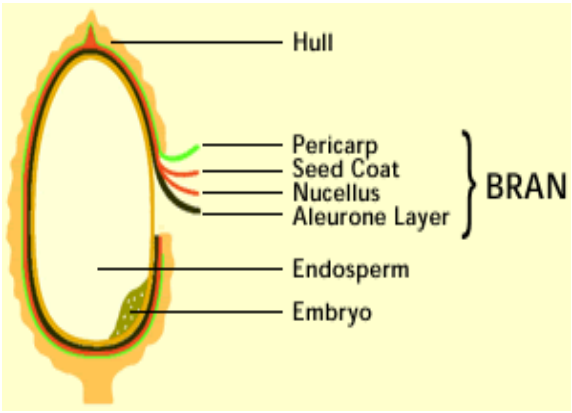


Figure 2.3 Rice grain structure (52)

2.7.1.1 Hull

Rice hulls, the hard outer covering brown rice which protect rice grain during growing season from insect and changing of external environment.

2.7.1.2 Brown rice or rice caryopsis

When the hull is removed, the brown rice can be seen. The brown rice consist of many layers such as following

2.7.1.3 Pericarp

The most outer fibrous layer which thick about 10 μm and locate the pigment in colored rice. Main faction of pericarp is to serve as a protective layer against molds and quality deterioration from oxidation and enzymes due to the movement of oxygen, carbon dioxide and vapor of water.

2.7.1.4 Seed coat or tegman

Next to the pericarp, seed coat is only a few cells in thickness 0.5 μm. This layer is rich in oil and protein contents but low in starch content.

2.7.1.5 Nucellus

Next to the seed coat is nucellus. This layer is 2.5 μm thick. There is bond between seed coat's cuticle and nucellus however, this bond is weak and tissue is easy to separating between this junctions.

2.7.1.6 Aleuronic layer or aleurone layer

Aleuronic layer is part of endosperm which is the most outer. Therefore, bran portion of rice grain comprise of pericarp, seed coat, nucellus and aleuronic layer and typically includes embryo. Rice bran contains 18-22 % oil. This part is removed from milling. In addition, bran is the most nutritious part of rice and rich in fiber, protein, oil, vitamins and mineral although it has very low starch.

2.7.1.7 Embryo or germ

Embryo is very small because weight only 1-3 % of total rice grain. It locates at central bottom portion of the grain. Embryo can grows to be a new plant because it is the living organism in the grain. Embryo is removed by milling result in indented shape at the end of the milled rice grain.

2.7.1.8 Endosperm

After removing the hull, the bran and the embryo, endosperm

remains. Endosperm is the largest portion of the rice grain because weight 80% of total rice grain. In addition, endosperm is rich in carbohydrate but low in vitamin and mineral.

2.8 Rice bran oil

2.8.1 Properties of rice bran oil

Rice bran oil made from pericarp, seed coat, nucellus, aleurone layer and embryo of the rice grain. It is highly nutritious oil and benefit because it contains many photochemical that have antioxidant activities such as vitamin E, γ-oryzanol, polyphenols and β-carotene (17-18), smoke point more than 200 °C which suitable to fry food. In addition, Rice bran oil is high in monounsaturated fatty acid (MUFA) and essential fatty acid contain oleic acid, linoleic acid and α-linolenic acid as unsaturated fatty acids, and stearic acids and palmitic as saturated fatty acid as showed in **Table 2.2** and composition of saponifiable lipids and unsaponifiable lipids in rice bran oil showed in **Table 2.3** (53-55). In addition, Phytosterols in rice bran oil include β-sitosterol, campesterol, stigmasterol, squalene and γ-oryzanol.

Table 2.2 Mainly fatty acid composition in rice bran oil and Riceberry bran oil in this study

Fatty acid composition (%)	**Rice bran oil**	**Riceberry bran oil**
Palmitic acid (16: 0)	21.5	18.6
Steric acid (18: 0)	2.9	2.8
Oleic acid (18: 1)	38.4	43.6
Linoleic acid (18: 2)	34.4	31.2
α-linolenic acid (18: 3)	2.2	1.2

Table 2.3 Composition of saponifiable lipids and unsaponifiable lipids in crude rice bran oil

Lipid	g/ 100 g oil
Saponifiable lipids	90-96
Neutral lipids	79-85
Monoacylglycerol	66-77
Diacylglycerol	2.4-3.6
Triacylglycerol	4.7-6.2
Free fatty acids	1.6-3.6
Waxes	2.4-3.6
Glycolipids	5.4-6.7
Phospholipids	3.6-4.8
Unsaponifiable lipids	4.2
4-Monomethylsterols	1.8
4-Desmethylsterols	0.4
4,4′- Dimethylsterols	1.2
Hydrocarbons	0.8
Tocopherol	0.04
Tocotrienol	0.07

2.8.2 Antioxidants in rice bran oil

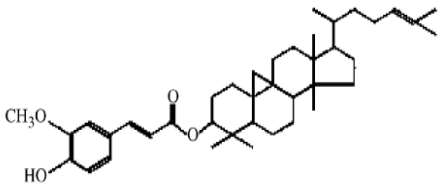
2.8.2.1 Phytosterols

Phytosterols or plant sterols are compounds which occur naturally and similar to cholesterol structure but have different side-chain configurations. Phytosterols are useful for pharmaceuticals which product therapeutic steroids, nutrition which anti-cholesterol additives in functional foods, anti-cancer properties and cosmetics manufacture. Phytosterols can be obtained from vegetable oils or from industrial wastes (56). Phytosterol are present in rice bran oil such as gamma-oryzanol and three group of phytosterol such as 4, 4′-dimethylsterols (1.2%), 4-monomethyl-sterols (0.4%) and 4-desmethylsterols (1.8%) (54). The esters of γ-

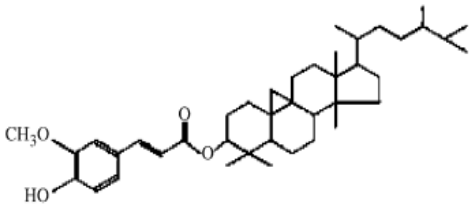
oryzanol consisted of two fractions. First is a polarity of ferulic acid which is stable and two is a functional group of alcohol compound including sterols and triterpene alcohols. γ-oryzanol in rice bran oil identify as a mixture of furulic acid esters of triterpene alcohols (57). In addition, γ-oryzanol in rice bran oil fraction contain 10 components following delta-7-stigmastenyl ferulate, stigmasteryl ferulate, cycloartenyl ferulate, 24-methylene cycloartanyl ferulate, delta-7-campestenyl ferulate, campesteryl ferulate, delta-7-sitostenyl ferulate, sitosteryl ferulate, campestanyl ferulate and sitostanyl ferulate. There are three compounds are main components of γ-oryzanol as follow cycloartenyl ferulate, 24-methylene cycloartanyl ferulate and campestanyl ferulate accounting for 80% of gamma-oryzanol in rice bran oil (58) (Figure 2.4). γ-oryzanol has antioxidant property which found that 24-methylene cycloartanyl ferulate is the highest antioxidant of γ-oryzanol (59). In addition, γ-oryzanol has demonstrated anti-hyperlipidemic property. From previous study, after feeding rice bran oil 10% level to rats for 8 weeks showed significantly lower in total plasma cholesterol (TC), low density lipoprotein cholesterol (LDL-C) and very low density lipoprotein cholesterol (VLDL-C) levels, both in cholesterol diet and free cholesterol diet. High density lipoprotein cholesterol (HDL-C) was increased. Liver cholesterol and plasma triglyceride (TG) reduced but no significant decreased while bile acid and neutral sterols were increased (60-61). Another study maintained rice bran oil 10% level to rat found that free esterified, TC and non HDL-C were significantly lower than those on 10% groundnut oil while HDL-C showed tendency to be higher and liver lipid were lower than 10% groundnut oil group. Addition of γ-oryzanol 0.5% level to diet with rice bran oil found a further significant decrease in TC level (62-63). In hypercholesterolemic rats after feeding cycloartenol, 24-methylene-cycloarthanol and phytosterol in amount present in rice bran oil unsaponifiable matter for 8 weeks found that cycloartenol alone significantly reduced both TC and TG levels (64).

Figure 2.4 Chemical structure of three main components of γ-oryzanol

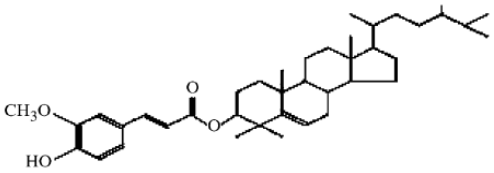
Cycloartenyl ferulate



24-methylene cycloartenyl ferulate



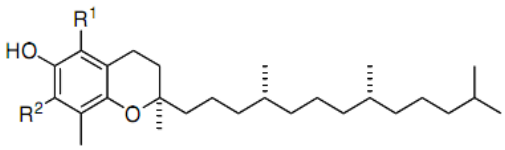
Campestanyl ferulate



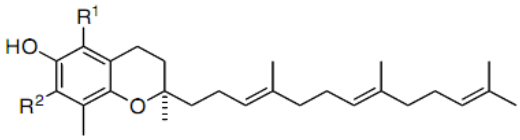
2.8.2.2 Vitamin E

Vitamin E is a name of molecule related compounds. These compound divide into two classes: first is tocopherol which have saturated side chains, second is tocotrienol which have unsaturated side chains. Each class composes of four vitamers that difference in number and location of methyl groups on the chromanol ring. Vitamers in both classes such as α, β, γ and δ. In addition, there is another compound such as all-rac α-tocopheryl acetate which use in food fortification (65). Vitamin E structures and the methyl groups showed in Figure 2.5

Figure 2.5 Chemical structure of tocopherol and tocotrienol of vitamin E



$R^1 = R^2 = CH_3$, α-Tocopherol
$R^1 = CH_3$, $R^2 = H$, β-Tocopherol
$R^1 = H$, $R^2 = CH_3$, γ-Tocopherol
$R^1 = R^2 = H$, δ-Tocopherol



$R^1 = R^2 = CH_3$, α-Tocotrienol
$R^1 = CH_3$, $R^2 = H$, β-Tocotrienol
$R^1 = H$, $R^2 = CH_3$, γ-Tocotrienol
$R^1 = R^2 = H$, δ-Tocotrienol

Vitamin E is found in plant and animal food. Plant foods, especially oil from plants considered the richest source of vitamin E. Tocopherols are found in leafy plant foods while tocotrienols are found in legume and cereal grains such as barley, oats and rice. The bran and germ sections are especially rich in tocotrienol (66-67). Vitamin E contents of oil per 100 g product including Riceberry bran oil in this study showed in **Table 2.4** (68).

Table 2.4 Vitamin E content of oil product (mg/100 g)

Natural oil	**Tocopherol**	**Tocotrienol**			
	α	**α**	**β**	**γ**	**δ**
Rice bran	32.4	23.6	NA	34.9	-
Riceberry bran	43.6	3.93	1.08	44.51	2.05
Palm	15	14.6	3.2	29.7	8
Wheat germ	133	2.6	18.1	NA	NA
Coconut	0.5	0.5	0.1	-	-
Soy bean	7.5	0.2	0.1	0	0
Olive	11.9	0	0	0	0

NA= data not available.

Major function of vitamin E is a lipid-soluble chain-breaking antioxidant. The process of lipid peroxidation or autooxidation found that tocopherol is the most effective chain-breakers. In addition, vitamin E has been proposed for a key role in delaying the pathogenesis of a variety of degenerative diseases such as inflammatory diseases, cancers, cardiovascular diseases, neurological disorders, cataract and in the maintenance of the immune system (35). Previous study determined effect of a

tocotrienol rich fraction obtained by molecular distillation from specially processed rice bran oil on cardiovascular disease risk factors on hypercholesterolemic human who total cholesterol (TC) > 220 mg/dl, a double blind 12-week study. After acclimation to an alcohol free regimen, all subjects were assigned to the National Cholesterol Education Program (NCEP) Step-1 diet (total fat <30% of total calories, saturated fat <19%, and cholesterol <300mg/day). After four weeks study period, 21 subjects was continued on the standard NCEP Step-1 diet, while a group of 20 added to diet 200 mg/day of a tocotrienol mix such as 12.5% α-tocotrienol, 21% γ-tocotrienol, 10% δ-tocotrienol, 4.5% β-tocotrienol, 17% $P_{2.5}$-tocotrienol and 18% unidentified tocotrienols. The result found that after 4 weeks of additional, the treated group had a significant decrease in plasma TC and low density lipoprotein-cholesterol (LDL-C) levels (-12% and -16%, respectively, versus -2% and -3% of the control group). In addition, significant decreases in lipoprotein (a), serum apolipoprotein B, platelet factor 4 and thromboxane B2 were observed in connection with the baseline level (-17%, -15%, -14% and –31% respectively) (69). Similarly, another study of Qureshi *et al.* (70) confirmed that a combined treatment with NCEP Step-1 diet and tocotrienol rich fraction of rice bran has also determined a 25% reduction in LDL-C plasma level of hypercholesterolemic subjects.

2.8.2.3 Polyphenol

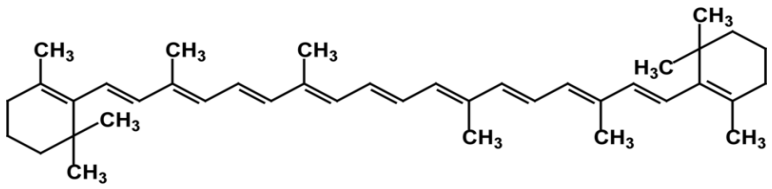
Polyphenol is the largest group of photochemical which distributed widely among the plant kingdom and an integral part in human diet. Dietary phenolics include phenolic acids, phenolic polymers and flavonoids (71-73). Phenolic acids form a diverse group such as hydroxycinnamic acids and hydroxybenzoic. Phenolic polymers are compounds of high molecular weight such as condensed tannins. Flavonoids compose the largest of plant phenol and over 6,000 difference flavonoids have been described (74). Polyphenols and flavonoids are powerful antioxidant properties. Plant polyphenols are various functions which act as singlet oxygen quenchers and as reduce agents as hydrogen atom-donating antioxidants. Some polyphenols also act as antioxidants owing to chelation of transition metal ion inhibit the Fenton and Haber-Weiss reaction which are important sources for active oxygen radicals. Flavonoids are effective scavenger of peroxyl and hydroxyl radicals and of the superoxide anion (75-79). In addition, both of polyphenol

and flavonoids inhibit low density lipoprotein (LDL) oxidation, aggregation and increase serum paraoxonase activity resulting in a further protection against cardiovascular disease (80). In rice, from the study of Chotimarkorn *et al.* (16) determined five varieties of long grain rice bran extract which are the most cultivated in Thailand from *Oryza Sativa L.* such as Khao Dawk mali 105, Khao Pathum Thani 60, Khao Suphan buri 90, Khao chinart 1 and Khao Gokho 13 found that all varieties have total phenolic content ranged from 2.2 to 3.2 mg gallic acid eq/ g rice bran and total flavonoid content ranged from 0.03 to 0.10 mg catechin eq/ g rice bran.

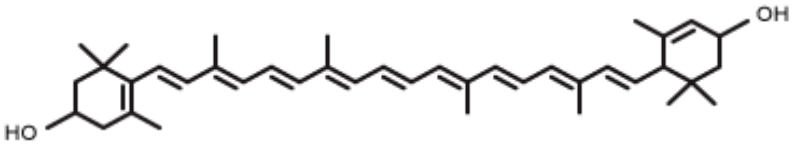
2.8.2.4 β-carotene and lutein

β-carotene and lutein are organic compound and type of carotenoids from more than 600 different carotenoids in nature. The basic structure of most carotenoids consists of a polyisoprenoid, C40 carbon chain with a series of conjugated, double located in the central portion of the molecule. β-carotene is dicyclic with β-ionone rings at both ends of molecule. β-carotene has internal molecule symmetry in which one half of molecule is same with the other half because central cleavage of β-carotene has the potential of producing two molecules of retinol. Lutein has oxygenated end groups. Chemical structure of beta-carotene and lutein showed in Figure 2.6.

Figure 2.6 Chemical structure of beta-carotene and lutein



Beta-carotene



Lutein

Carotenoids are important organic pigments which occur naturally in plants, algae and some photosynthesis organism. The color ranks from reddish -brown to deep-violet

but less than 10% of these pigments have vitamin A activity. β-carotene are classified as carotene while lutein as xanthophylls. The pigment with the greatest vitamin A activity is β-carotene (81). β-carotene in human diet act as vitamin A precursor because β-carotene can be convert in the intestinal tract. Carotenoids present in the interior membranes of cells as well as in lipoprotein. In plant tissue, carotenoid has function importantly as supplementary pigments in photosynthetic process and photoprotection. The conjugated polyene structure of carotenoids allows molecule to absorb light. The scavengers of reactive oxygen species, especially singlet molecular oxygen and peroxyl radicals which can quench excited tripet states ($^3O_2$), an important property for the photoprotective effect in plants (82). In addition, carotenoids are effective antioxidant which can react with free radicals and quench or inactive singlet oxygen. Carotenoids are efficient to protect lipids from autoxidation of free radical by react directly with peroxyl radicals. Carotenoids such as β-carotene produced a carbon center carotenyl radical (β-car)$^\bullet$ then, inhibit propagation and promote termination of oxidation chain reaction (83-85)

$$\text{β-carotene} + \text{ROO}^\bullet \longrightarrow \text{ROO-(β-car)}^\bullet$$
$$\text{ROO-(β-car)}^\bullet + \text{ROO}^\bullet \longrightarrow \text{Inactive product}$$

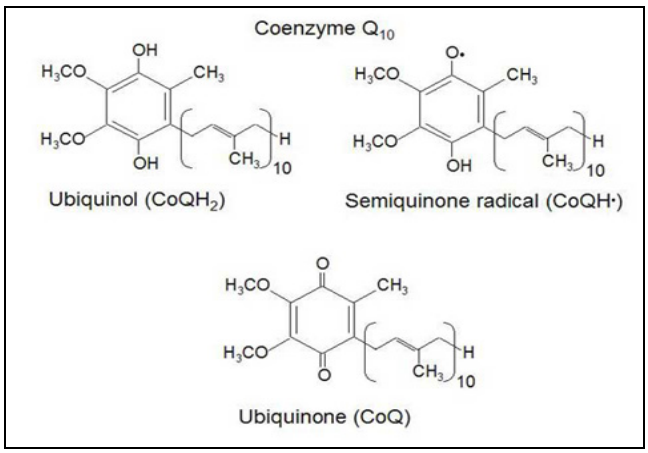
The antioxidant activity of β-carotene is faster than lutein. Studies suggest that β-carotene works synergistically with vitamin E in scavenging radicals and inhibiting lipid peroxidation, although vitamin E has higher reactivity toward peroxyl radical than β-carotene (86). β-carotene is function in interior of the membrane, whereas α-tocopherol of vitamin E functions on or at the surface of membrane (87). In addition, β-carotene supplementation helps to make the LDL more resistant to metal-induced lipid oxidation (88). Epidemiological studies have shown that those individuals with high intakes of fruits and vegetables, which are also rich in carotenoids, of those individual with higher serum carotenoid concentrations have lower incidence of disease such as cardiovascular disease and cancer (89-93). There are the study in traditional subsistence in the Philippines upland region found that the highest contents of β-carotene dependent on the pericarp color which found in the black and purple varieties (0.05 μg/g dry matter), follow by the reddish and brown rice (0.02 μg/g dry

matter), and the lowest contents were found in the colorless varieties (0.01 µg/g dry matter) and the high yielding varieties (0.01 µg/g dry matter). There are only the black and purple varieties on a statistically significant level difference from the other classes. In addition, there are the β-carotene biosynthesis pathway into rice endosperm through genetic engineering has report led to contents 1.6 µg/g even in milled rice (94-95) while Chanphrom *et al.* (17) found that β-carotene content of pigmented rice varieties in Thailand ranged from 0.02 to 0.91 µg/g dry basis while in Riceberry bran found the contents of β-carotene was 0.16 µg/g dry basis. In addition, Riceberry bran oil in this study found β-carotene content ranged from 3.96 to 4.34 µg/g.

2.8.2.5 Coenzyme Q_{10}

Coenzyme Q_{10} or Ubiquinone is the molecule contains one quinine ring with 10 isoprenyl units. Chemical structure of coenzyme Q_{10} showed in Figure 2.7

Figure 2.7 Chemical structure of coenzyme Q_{10} (96)



CoQ_{10} play an important role as electron and proton transporter in the mitochondria for ATP production for all of organisms such as microorganisms, plants, animals including human. CoQ_{10} exists both in an oxidized (Ubiquinone, CoQ) and a reduced form (Ubiquinol, $CoQH_2$). Thus, CoQ_{10} has a role as a lipid soluble antioxidant. Many studies found that after CoQ_{10} supplementation, CoQ_{10} reduced low density lipoprotein (LDL) oxidation both *in vitro* and *vivo* (97-98). In addition, CoQ_{10} can recycle α-tocopherol of vitamin E from its radical in lipoprotein and membrane.

CoQ_{10} obtain from synthesis in the body and diet. However, CoQ_{10} and cholesterol share the same biosynthesis pathway. When using 3-hydroxy-3-methyl-glutaryl-CoA reductases (HMG-CoA-R) which inhibit cholesterol synthesis, it is likely to affect the metabolism and physiological function of CoQ_{10} (99). In addition, CoQ_{10} was used in commercially as food supplement and cosmetics. CoQ especially accumulates in the bran and germ of rice. There are the latest study in Japan that produced CoQ_{10}-enriched rice plants by introduced the gene encoding the enzyme decaprenyl diphosphate synthase for the synthesis of 10 isoprene units for the CoQ side chain into rice *Sugary* and *Shrunken* (100). The result showed smaller endosperm and consequence with a larger weight ratio of bran and germ and CoQ_{10} contents were 34.5 ± 15.3 μg/g in *Sugary* and 28.1 ± 14.3 μg/g in *Shrunken* while CoQ_{10} contents in other rice such as *Nipponbare* were 12.2 ± 0.3 and 10.2 ± 2.2 μg/g respectively (101).

2.9 Rice bran oil and antioxidant property

Revilla *et al.* (102) was evaluate antioxidant and hypocholesterolemic activities of a water-soluble enzymatic extract from rice bran (EERB) which contains water soluble form such as tocopherols, tocotrienols, gamma-oryzanol, sterols, and peptides. The result showed that EERB specifically against lipid oxidation by inhibit the generation of thiobarbituric acid reactive substances (TBARS) and lipid peroxides which was determined in emulsion prepared with fish oil that contain fatty acid very susceptible to oxidation. In addition, the hypocholesterolemic activity of EERB was evaluated in male wistar rats through two different hypercholesterolemic diet models: model I: received 1% cholesterol, 0.5% of cholic acid and EERB for 3 months and model II: received 0.30 % cholesterol and EERB for 4 weeks. The result showed that both group had a reduction in total cholesterol and increase in HDL-cholesterol levels.

Kanaya *et al.* (103) determined anti-oxidative effects of crude lipophilic rice bran extract (Ricetrienol®) which contains α-tocopherol, tocotrienol and phytosterol in obese diabetic KKAy mice. KKAy mice received normal diet (DM group) or a diet including 0.1% Ricetrienol® (RT group), and non-diabetic C57BL mice (C group). After 6 weeks study period, lipids, malondialdehyde (MDA), α-

tocopherol, body weight, glutathione peroxidase 1 (GSH-Px) mRNA expression, plasma glucose,HbA1c in the kidney were measured while at 1 week and at 6 weeks, urine 8-isoprostane and 8-hydroxy deoxyguanosine (8-OHdG) were also measured. The result showed that Ricetrienol® did not affect body weight, hyperglycemia or hyperlipidemia. While Plasma MDA, 8-OHdG and urine 8-isoprostane in DM group were significantly increased compared with C group and the elevation of plasma MDA was significantly suppressed by Ricetrienol®. In addition, GSH-Px mRNA expression in RT group was significantly increased when compared with C group. These results suggest that Ricetrienol® exerts a protective effect against oxidative damage in diabetes mellitus.

The probability of a sensitive biomarker of oxidative DNA damage and oxidative stress is 8-hydroxy-2′-deoxyguanosine (8-OHdG) while reactive oxygen species (ROS) which is oxidative stress and have been reported to be a cause of diabetes in experimental animals induced by chemicals such as streptozotocin (STZ). Thus, Hsieh *et al.* (104) determined oxidative DNA damage in multiple tissues in rats with STZ-induced diabetes that treat with or without rice bran oil by measured the levels of 8-OHdG in mitochondrial DNA (mtDNA) in the liver, kidney, pancreas, brain, and heart. There were significant reductions in mtDNA 8-OHdG levels in the liver, kidney, and pancreas of diabetic rats treated with rice bran oil compared with diabetic rats without rice bran oil. This result demonstrated that oxidative mtDNA damage may occur in multiple tissues of STZ-induced diabetics rats. Treatment with rice bran oil may converse the increase in frequency of 8-OHdG.

2.10 Rice bran oil and antihyperlipidemic property

Sharma and Rukmini demonstrated that after feeding rice bran oil 10% level to rats for 8 weeks showed significantly lower in total plasma cholesterol (TC), low density lipoprotein cholesterol (LDL-C) and very low density lipoprotein cholesterol (VLDL-C) levels, both in cholesterol diet and free cholesterol diet. High density lipoprotein cholesterol (HDL-C) was increased. Liver cholesterol and plasma triglyceride (TG) reduced but no significant decreased while bile acid and neutral sterols were increased (60-61).

In Purushothama *et al.* (105) study effect of feeding two levels of rice bran oil such as 5% and 20% of the diet were compared with fed by peanut oil in wistar rats on growth, plasma liver and lipid parameters. There was no significant difference to the organ weights between control and experimental groups. In rice bran oil group had lower levels of total cholesterol (TC), triglyceride (TG) and phospholipids (PL). In addition, in wistar rats that received 20% rice bran oil of their diet had an increase of 20% in high density lipoprotein-cholesterol (HDL-C) when compared to the wistar rats fed with peanut oil within 18 weeks ($p<0.05$). Similarly, low density lipoprotein-cholesterol (LDL-C) and very low density lipoprotein-cholesterol (VLDL-C) were lower in rice bran oil groups than in the peanut oil groups. However, there was no significant difference in the plasma cholesterol/phospholipid ratio or in the polyunsaturated/saturated fatty acids ratio, or any in oleic/stearic, oleic/linoleic palmitoleic/palmitic, oleic/palmitic, and oleic/palmitoleic ratios between the two groups.

In another study were advised twelve moderately non-obese hyperlipoproteinemic subjects who had mean baseline of plasma total cholesterol (TC) and plasma triglyceride (TG) 247.3±10.55 and 349.8±42.41 mg/dl, respectively to replacement normally cooking oils that they used with rice bran oil compared with those of eleven subjects with a similar baseline lipid pattern. The result found that in rice bran oil group showed 16% and 25% decrease in plasma total cholesterol (TC) and 32% and 35% reduction of plasma triglyceride (TG) after 15 and 30 days of administration respectively when compared to control group. In addition, the decrease in lipid levels was faster in subjects with higher baseline levels (106).

Cicero *et al.* (107) studied in a 9 year old Caucasian patient who affected by Fredrickson type IIb, type of dyslipidemia that is genetic disorder with total cholesterol (TC) >200 and triglyceride (TG) >200mg/dl. After treatment with rice bran oil 20 g/day and polyunsaturated fatty acids 650 mg/day for 1 month, the result showed significant decrease of TC, low density lipoprotein-cholesterol (LDL-C), TG and lipoprotein (a) reaching standard values of her age.

CHAPTER III
MATERIALS AND METHODS

3.1 Animals

Mice weighed 20-25 g. (aged ~ 3 months) were divided by genotypes into 2 major groups, wild type or C57BL/6J or $m\alpha^{+\,+}/m\beta^{+\,+}$ (normal) and heterozygous β-globin gene knocked-out mice or $m\alpha^{++}/m\beta^{+-}$ (β-thalassemic) mice from Thalassemia Research Center, Institute of Molecular Biosciences, Mahidol University. All mice were housed in:

Cage and Number of animals per cage: polystylene with stainless steel wire-bar lid 1-3 mice per small cage and 2-4 mice per large cage.

Controlled environment: temperature range at about 25±1 °C, relative humidity range of about 40-50 %, light: dark cycle 12:12 hours.

Feed: All mice were fed on the commercial diet (No. CP 082) product of Perfect Companion Group Co. Ltd. Food was provided in stainless steel wire-bar lid feeder ad libitum.

Water: The reverse osmosis (RO) water was used for drinking water which was chlorinated at a concentration of 10-12 ppm. to make it free from *Pseudomonas aeruginosa*. The drinking water was provided in bottles with sipper tubes.

Bedding: Hard wood chips were used for mice as bedding material.

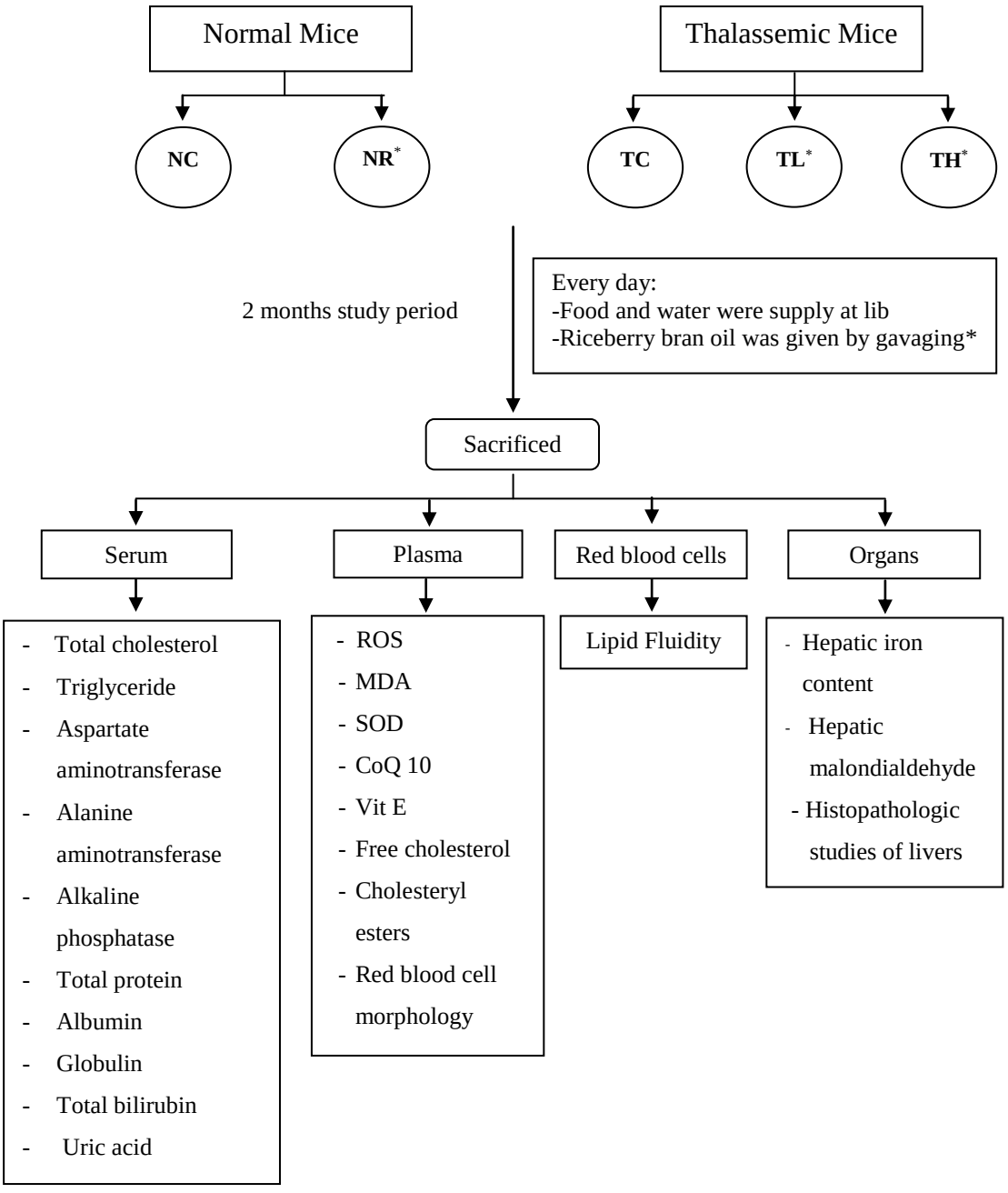
Monitor: All of mice were weighed every 2 days.

3.2 Experimental study (Figure 3.1)

Normal mice consisted of 2 subgroups such as 14 control normal mice group (NC) and 14 normal mice receiving high dose group (NR).

β-thalassemic mice consisted of 3 subgroups such as 10 control thalassemic mice group (TC), 10 thalassemic mice receiving low dose group (TL) and 10 thalassemic mice receiving high dose group (TH).

Figure 3.1 Flow chart for study design



3.3 Riceberry bran oil

Riceberry bran oil obtained from Rice Science Research Center, Kasetsart University, Kamphangsaen, Nakhon Pathom, Thailand was extracted from Riceberry bran at Thanachart natural oil and product, Saraburi, Thailand by supercritical fluid extraction method (SFE). Supercritical fluid extraction method (SFE) is a new

technique for extracting rice bran oil using CO_2 as the extraction solvent. Riceberry rice was a variety developed by crossed breeding of Khao Dawk Mali 105 from Rice Research Institute and Chao Hom Nil from Kasetsart University. The antioxidant properties of Riceberry bran oil is shown in **Table 3.1**

Table 3.1 Antioxidant contents in Riceberry bran oil (18)

Gamma-oryzanol (mg/g)	**Beta carotene** (µg/g)	**Lutein** (µg/g)	**Vit E** (mg/g)	**Polyphenol** (mg/g)	**CoQ_{10}** (µg/g)
14	22.94	14.64	0.92	0.46	20

Antioxidant activity (18)

- Oxygen radical absorbance capacity (ORAC): 215.13 (lipophilic) µmoles Trolox/g
- Ferric reducing antioxidant power (FRAP): Not detectable in Riceberry bran oil
- Free of heavy metal (Cadmium and Plumbum)

3.3.1 Doses of Riceberry bran oil for gavaging

3.3.1.1 Low dose for TL group (thalassemic mice receiving low dose)

Riceberry bran oil for TL group was derived from the previous study (108-110). Low dose for gavaging was 17 mg/20 g body weight/day/mice or equal to 50 g/60 kg. body weight/day in human or 3 tablespoons of fat which is not exceed the daily recommendation (65 g per day or about 4 tablespoons) (111).

3.3.1.2 High dose for NR group (normal mice receiving high dose) and TH group (thalassemic mice receiving high dose)

Double amount of Riceberry bran oil was used for high dose for gavaging. It was 36 mg/ 20 g body weight/day/mice or equal to 109 g/60 kg. body weight/ day in human or about 7 tablespoons.

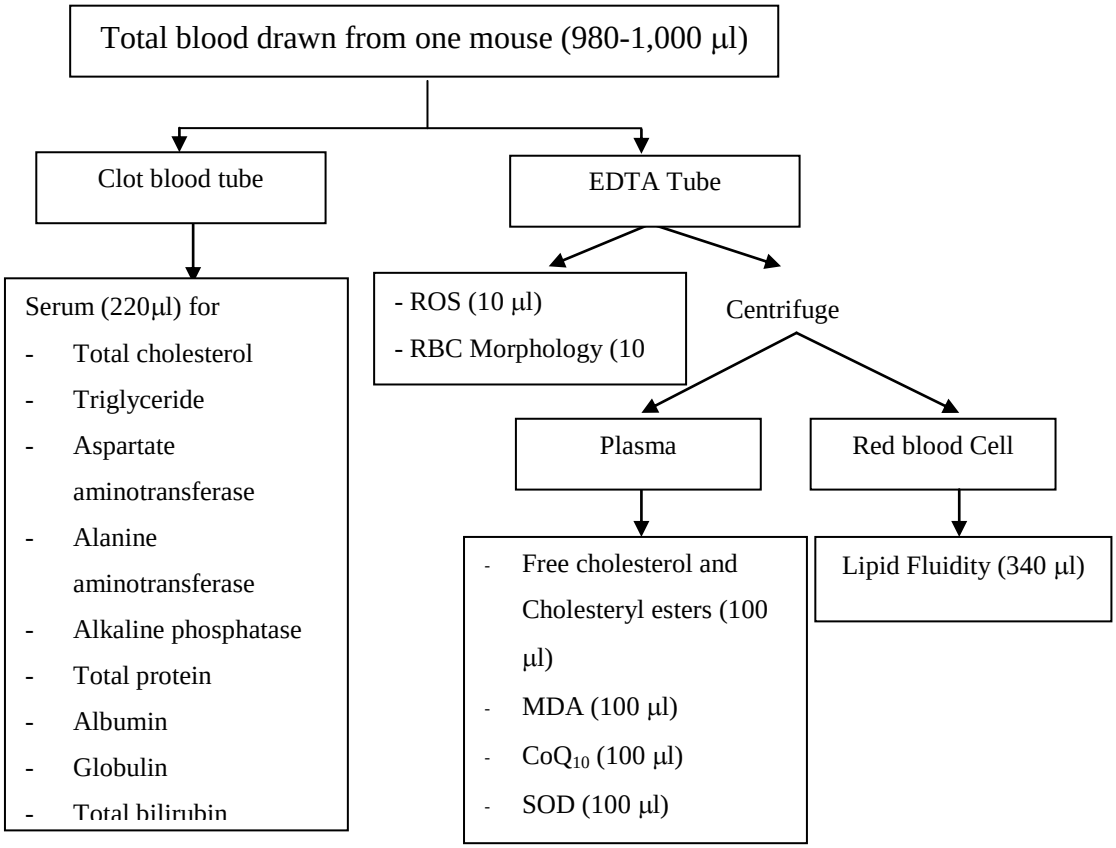
3.3.2 Quality control of Riceberry bran oil

Riceberry bran oil using in this study was manufactured in one batch, thoroughly mixed and aliquot of Riceberry bran oil in dark brown microcentrifuge tubes. These samples were kept in a dark dry place, below 4 °C. Iron content was also determined in Riceberry bran oil. The verification of antioxidant activities and antioxidant contents of Riceberry bran oil were done twice, before and at the end of the study.

3.4 Blood and organs collection

After 2 months study period, all of mice were anesthesia by intraperitoneal injection with Zoletil 100 (Tiletamine and Zolazepam) 30 mg./kg. Blood was collected from heart and separated into 2 microcentrifuge tubes for clotted blood and heparinized blood. Plasma was separated from red blood cells by centrifugation at 2,300 g at 4 °C for 10 minutes and stored in dark brown microcentrifuge tube at -80 °C before analysis. Red blood cells were analyzed within 24 hour after collection. Serum was stored at -4 °C before analysis. Amount of blood for all parameters assessment are shown in **Figure 3.2** Organs: heart, lung, liver, spleen, left and right kidney were weighed, washed in normal saline and kept in aluminum foil at -80°C for further analysis.

Figure 3.2 Blood for all parameters assessment



3.4.1 Parameters

In this study, plasma, red blood cells, serum and organs were determined by measuring:

3.4.1.1 Oxidative stress status and antioxidants

- Reactive Oxygen Species (ROS) was analyzed by flow cytometry-based 2′ 7′ dichlorofluorescein assay at Thalassemia Research Center, Institute of Molecular Biosciences, Mahidol University (112). Detail of analysis is shown in Appendix A.
- Lipid fluidity was analyzed by Electron Spin Resonance (ESR) Spin Labeling method at Department of Pharmacology, Faculty of Science, Mahidol University (113). Detail of analysis is shown in Appendix B.

- Malondialdehyde (MDA) was analyzed by spectrofluorometry at Department of Pharmacology, Faculty of Science, Mahidol University (114). Detail of analysis is shown in Appendix C.
- Superoxide dismutase (SOD) was analyzed by Superoxide Dismutase Activity kit at Institute of Nutrition, Mahidol University (115). Detail of analysis is shown in Appendix D.
- CoQ_{10} was analyzed by High Performance Liquid Chromatography with electro-chemical detector (HPLC-ECD detector) at Kobe Gakuin University, Japan (116). Detail of analysis is shown in Appendix E.
- Vitamin E was analyzed by High Performance Liquid Chromatography (HPLC) (Technical Service, Institute of Nutrition, Mahidol University) (117). Detail of analysis is shown in Appendix F.

3.4.1.2 Biochemical assessment

- Free cholesterol (FC) and cholesteryl esters (CEs) were analyzed by High Performance Liquid Chromatography (HPLC) at Department of Pharmacology, Faculty of Science, Mahidol University (118). Detail of analysis is shown in Appendix G.
- Total cholesterol (TC), triglyceride (TG), cholesteryl esters (CEs), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALK), total protein, albumin, globulin, total bilirubin and uric acid were analyzed by Automatic Analyzer at Center of laboratory medicine, Faculty of Medical Technology, Mahidol University.

3.4.1.3 Hematological assessment

- Red blood cell morphology was analyzed at Thalassemia Research Center, Institute of Molecular Biosciences, Mahidol University (119). The method of staining is shown in Appendix H.

3.4.1.4 Pathological assessment

- Hepatic malondialdehyde was analyzed by spectrofluorometry at Department of Pharmacology, Faculty of Science, Mahidol University (114). Detail of analysis is shown in Appendix C.
- Hepatic iron content was analyzed by spectrophotometer (Thalassemia Research Center, Institute of Molecular Biosciences, Mahidol University) (120). Detail of analysis is shown in Appendix I.
- Histopathologic studies of livers were analyzed at Thalassemia Research Center, Institute of Molecular Biosciences, Mahidol University (121).

3.5 Ethical Approval

This study was obtained ethical approval by the Mahidol University Animal Ethics Committee.

3.6 Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Science/Personal Computer (SPSS/PC) for windows version 15. Data were expressed as mean ± SD. Comparison between two groups was analyzed by independent sample t-test for parametric data and Mann Whitney test for non parametric data. Comparison among group was analyzed by one way analysis of variance (ANOVA) with Fisher's protected least significant different test and Kruskal-Wallis test for parametric data and non parametric data. Relationship of data was analyzed by Spearman's rho correlation. Results were considered significant when p value < 0.05.

CHAPTER IV
RESULTS

4.1 General information of mice in the study

Total number of mice in the study were 58 which had been divided by genotypes into 2 major groups, 28 wild type mice or C57BL/6J mice or $m\alpha^{++}/m\beta^{++}$ (normal) mice and 30 heterozygous β-globin gene knocked-out mice or $m\alpha^{++}/m\beta^{+-}$ (β-thalassemic) mice**.**

Normal mice consisted of 2 subgroups: 14 control normal mice group (NC) and 14 normal mice receiving high dose of Riceberry bran oil group (NR).

β-thalassemic mice consisted of 3 subgroups: 10 control thalassemic mice group (TC), 10 thalassemic mice receiving low dose of Riceberry bran oil group (TL) and 10 thalassemic mice receiving high dose of Riceberry bran oil group (TH).

General characteristics of mice in the study were shown in **Table 4.1**. Age and weight at the beginning of the study in all groups were no any statistically difference.

At the 1st month of the study, Weight of mice in all groups increased from the weight at the beginning of the study (month 0), except group TL and TH which were decreased. However, there was no any statistically significant differences in all groups of mice when comparing weight between the weight at the beginning (month 0) and the 1st month of the study. Moreover, there was also no any statistically significant difference when comparing the weight of mice in the 1st month within normal mice group, β-thalassemic mice group and among normal and β-thalassemic mice group.

However, at the 2nd month of the study, the weight of mice in all groups were increasing from the 1st month and had a statistically significant differences in the group of NC, NR, TC, and TH at $P<0.01$, $P<0.01$, $P<0.01$, and $P<0.05$ respectively. When comparing the weight of mice at the 2nd month among all groups of mice, the weight of mice in control normal group (NC) was higher than the β-Thalassemic mice

group receiving high dose (TH) with statistically significant at $P<0.05$. Moreover, when comparing the weight of mice between the weight at the beginning (month 0) and the 2nd month of the study the result found that there were a statistically significant differences in the group of NC, NR, TC, and TH at $P<0.01$, $P<0.05$, $P<0.01$, and $P<0.05$ respectively.

Table 4.1 Age at the beginning of the study and body weight of mice (g)

Groups / Month	Normal mice		β-Thalassemic mice		
	NC (N=14)	**NR (N=14)**	**TC (N=10)**	**TL (N=10)**	**TH (N=10)**
Age at 0	3.3 ± 1.1	3.4 ± 1.0	3.6 ± 1.6	3.4 ± 1.4	3.6 ± 1.4
0	22.8 ± 2.6	22.9 ± 2.7	21.6 ± 2.2	23.5 ± 2.6	21.6 ± 2.8
1	23.5 ± 1.9	23.1 ± 2.6	22.4 ± 2.3	22.9 ± 2.6	21.3 ± 2.5
2	25.0 ± 2.1[a,b]	24.0 ± 2.6 [aa,b]	24.1 ± 2.8 [a,b]	23.8 ± 2.4	22.4 ± 2.4 [aa,bb,c]

All values are mean ± SD.

Significantly different from Month 0: [a] $P<0.01$, [aa] $P<0.05$.

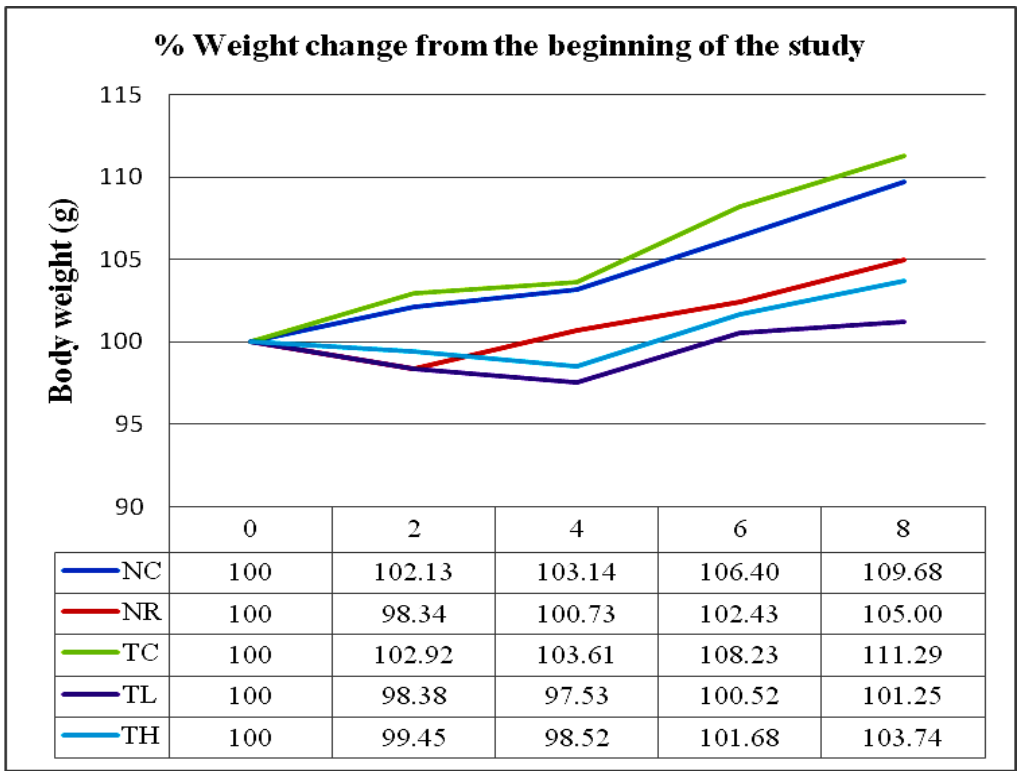
Significantly different from Month 1: [b] $P<0.01$, [bb] $P<0.05$,

[c] Significantly different from NC group: $P<0.05$.

NC=Control normal mice; NR=Normal mice receiving high dose;

TC=Control thalassemic mice; TL=Thalassemic mice receiving low dose and TH=Thalassemic mice receiving high dose

Figure 4.1 % Weight change from the beginning of the study (g/wk)



	0	2	4	6	8
NC	100	102.13	103.14	106.40	109.68
NR	100	98.34	100.73	102.43	105.00
TC	100	102.92	103.61	108.23	111.29
TL	100	98.38	97.53	100.52	101.25
TH	100	99.45	98.52	101.68	103.74

NC=Control normal mice; NR=Normal mice receiving high dose;
TC=Control thalassemic mice; TL=Thalassemic mice receiving low dose and TH=Thalassemic mice receiving high dose

Figure 4.1 shows % weight change from the beginning of the study (g/wk). Weight change was calculated as percentage of the original weight measured at the beginning of the study (week 0) which equal to 100%.

4.2 Effect of Riceberry bran oil consumption in normal and β-thalassemic mice

After 2 months of daily gavaging of Ricebery bran oil, mice were sacrificed and the following parameters were evaluated.

4.2.1 Oxidative stress status and antioxidants

4.2.2 Biochemical assessment

4.2.3 Hematological assessment

4.2.4 Pathological assessment

4.2.1 Oxidative stress status and antioxidants

Parameters included oxidative stress status, enzymatic and non-enzymatic antioxidant activities. Oxidative stress levels were measured via reactive oxygen species (ROS) level, lipid fluidity and malondialdehyde (MDA). Superoxide dismutase (SOD) as enzymatic antioxidant and coenzyme Q_{10} (CoQ_{10}) and vitamin E. as non-enzymatic antioxidants were evaluated. The result showed that

4.2.1.1 Oxidative stress status

4.2.1.1.1 Reactive oxygen species (ROS) level

Reactive oxygen species (ROS) level of normal and β-thalassemic mice in this study were determined by flow-cytometry based 2'7' DCFH-DA assay. Reactive oxygen species level or Mean Fluorescence intensity (MFI) generated from the reaction of oxidation between DCF and ROS under H_2O_2 stimulation for 20 minutes. The results were measured and interpreted in **Table 4.2**.

According to **Table 4.2**, reactive oxygen species level of mice in TC group was statistically significant higher than of mice in NC group ($P<0.05$). This represented that there was a higher reactive oxygen species level in β-thalassemic mice when compared to normal mice. Furthermore, after the β-thalassemic mice-TL group receiving rice bran oil, the reactive oxygen species level decreased compared to the TC group, while TH group was increased. In normal mice, after receiving rice bran oil (NR group) the reactive oxygen species level decreased when compared to NC group, but without any statistically significant difference.

Table 4.2 Reactive Oxygen Species by flow cytometric analyses of the fluorescence of H_2O_2-stimulated at min 20 of red blood cells in mice

Groups / ROS	Normal Mice		β-Thalassemic mice		
	NC (N=5)	**NR** (N=5)	**TC** (N=4)	**TL** (N=3)	**TH** (N=3)
At min 20	68.2 ± 14.5	64.9 ± 17.0	193.2 ± 39.1 [a]	165.9 ± 20.5	201.2 ± 47.6

All values are mean ± SD.

[a] Significantly different from NC group: $P<0.05$

NC=Control normal mice; NR=Normal mice receiving high dose; TC=Control thalassemic mice; TL=Thalassemic mice receiving low dose and TH=Thalassemic mice receiving high dose

4.2.1.1.2 Lipid fluidity

Lipid fluidity of red blood cell membrane was measured by Electron Spin Resonance (ESR) spectroscopy using spin labelling technique with two spin probe such as 5-doxyl stearic acid (5-DS) and 16-doxyl stearic acid (16-DS). The results of lipid fluidity near the area of phospholipids head group as measured by 5-DS showed that order parameter (S) was no significantly difference within normal mice, β-thalassemic mice and among normal and β-thalassemic mice (**Table 4.3**). Regarding to lipid fluidity in the area of hydrophobic region of red blood cell membranes as measured by 16-DS showed that h_0/h_{-1} in the group of β-thalassemic mice improved after receiving Riceberry bran oil (The decreased of h_0/h_{-1} indicated order parameter ↓, fluidity ↑ and rigidity ↓). The h_0/h_{-1} value in control β-thalassemic mice group (TC) was significantly higher (lower rate of fluidity) when compared to that of the control of normal mice group (NC) ($P<0.05$). After receiving rice bran oil, h_0/h_{-1} in β-thalassemic mice in TL and TH groups decreased to a level closed that of NC group. Furthermore, TL group had significantly lower h_0/h_{-1} than that of the TC group ($P<0.05$). For other parameters, there was no significantly difference in τ_B and τ_C

Table 4.3 Parameters for lipid fluidity at the different regions in red blood cell membranes in mice plasma

Groups / Parameters	Normal mice		β-Thalassemic mice		
	NC (N=9)	**NR** (N=9)	**TC** (N=6)	**TL** (N=7)	**TH** (N=7)
Order parameter (S)	0.71 ± 0.01	0.71 ± 0.01	0.71 ± 0.01	0.72 ± 0.01	0.71 ± 0.02
τ_B ($\times 10^{10}$ s)	18.5 ± 1.4	18.1 ± 1.2	18.1 ± 1.0	18.0 ± 1.2	17.8 ± 1.0
τ_C ($\times 10^{10}$ s)	22.7 ± 1.4	22.3 ± 0.9	23.2 ± 1.3	22.5 ± 1.2	22.4 ± 1.0
Order parameter (h_0/h_{-1})	4.41 ± 0.22	4.41 ± 0.16	4.70 ± 0.04 [a]	4.52 ± 0.19 [b]	4.61 ± 0.18

All values are mean ± SD.

[a] Significantly different from NC group: $P<0.05$, [b] Significantly different from TC group: $P<0.05$.

NC=Control normal mice; NR=Normal mice receiving high dose; TC=Control thalassemic mice; TL=Thalassemic mice receiving low dose and TH=Thalassemic mice receiving high dose

4.2.1.1.3 Malondialdehyde

The product of lipid peroxidation was malondialdehyde (MDA). MDA level was the reaction of thiobarbituric acid (TBA) test by using spectrofluorometer 515 nm excitation and 553 nm emission filters. The results were shown in **Table 4.4** which found that there was no statistically significant difference within normal mice and β-thalassemic mice. However, MDA level was statistically significant decreasing after β-Thalassemic mice group has received rice bran oil in high dose (TH) when compared to the control normal mice group (NC) ($P<0.05$). Furthermore, MDA level of normal mice in NR group decreased 7% from NC group similarly as β-Thalassemic mice in TL and TH group which MDA level decreased 15% and 31% respectively, when compared to TC group.

Table 4.4 The level of malondialdehyde (MDA) in mice (µmol/L)

Groups / Parameter	Normal Mice		β-Thalassemic mice		
	NC (N=12)	**NR** (N=11)	**TC** (N=8)	**TL** (N=9)	**TH** (N=8)
MDA (µmol/L)	0.014 ± 0.004	0.013 ± 0.004	0.013 ± 0.006	0.011 ± 0.005	0.009 ± 0.004 [a]

All values are mean ± SD.

[a] Significantly different from NC group: $P<0.05$

NC=Control normal mice; NR=Normal mice receiving high dose; TC=Control thalassemic mice; TL=Thalassemic mice receiving low dose and TH=Thalassemic mice receiving high dose

4.2.1.2 Enzymatic antioxidant

4.2.1.2.1 Superoxide dismutase

Superoxide dismutase was the primary defense mechanism against free radical of body which converted superoxide radical to hydrogen peroxide and ground state oxygen. SOD analyzed by using enzymatic assay kits (Biovision Laboratory, Switzerland). The results were shown in **Table 4.5** which found that there was no any significantly difference within normal mice, β-thalassemic mice and among normal and β-thalassemic mice.

Table 4.5 Superoxide dismutase in plasma of mice (U/ml)

Groups / Parameter	Normal mice		β-Thalassemic mice		
	NC (N=6)	**NR** (N=6)	**TC** (N=4)	**TL** (N=4)	**TH** (N=4)
SOD (U/ml)	9.2 ± 5.8	7.5 ± 6.9	16.2 ± 4.8	18.8 ± 2.5	12.5 ± 8.7

All values are mean ± SD.

NC=Control normal mice; NR=Normal mice receiving high dose; TC=Control thalassemic mice; TL=Thalassemic mice receiving low dose and TH=Thalassemic mice receiving high dose

4.2.1.3 Non-enzymatic antioxidants

4.2.1.3.1 Total CoQ_{10} content

Total CoQ_{10} content (total amount of CoQ_{10} that had been oxidized and reduced) was analyzed by using HPLC-ECD (high performance liquid chromatography with electrochemical detector). The result (**Table 4.6**) showed that β-Thalassemic mice in the TC group had total CoQ_{10} content lower than normal mice in NC group with statistically significant (P<0.05). After receiving rice bran oil, the level of CoQ_{10} in NR group was significantly increased (P<0.05) compared to the NC group. Similarly, in the group of β-Thalassemic mice after receiving rice bran oil, total CoQ_{10} content increased both TL and TH group compared to TC group which statistically significant in the TH group. However, total CoQ_{10} content in TL and TH group had no statistically significant differences when compared to TC group and NC group.

Table 4.6 Total CoQ_{10} content in mice plasma (ng/ml)

Groups / Parameter	Normal mice		β-Thalassemic mice		
	NC (N=9)	**NR** (N=9)	**TC** (N=6)	**TL** (N=7)	**TH** (N=7)
CoQ_{10} (ng/ml)	25.9 ± 4.5	32.0 ± 4.3 [a]	17.6 ± 2.9 [a]	21.2 ± 3.6	22.0 ± 3.9 [b]

All values are mean ± SD.

[a] Significantly different from NC group: P<0.05,

[b] Significantly different from TC group: P<0.05

NC=Control normal mice; NR=Normal mice receiving high dose; TC=Control thalassemic mice; TL=Thalassemic mice receiving low dose and TH=Thalassemic mice receiving high dose

4.2.1.3.2 Vitamin E

Vitamin E levels were analyzed using HPLC (high performance liquid chromatography). The result as shown in **Table 4.7**, which found that there was no any significantly difference within normal mice, β-thalassemic mice and among normal and β-thalassemic mice. However, vitamin E levels in plasma of β-Thalassemic mice after receiving rice bran oil in TL and TH group had been increasing 3% and 7% respectively when compared to vitamin E levels in TC group. The vitamin E levels of normal mice after receiving rice bran oil in NR group had been decreasing when compared to NC group.

Table 4.7 Vitamin E levels in mice plasma (µg/dl)

Groups / **Parameter**	**Normal mice**		**β-Thalassemic mice**		
	NC (N=12)	**NR** (N=12)	**TC** (N=6)	**TL** (N=6)	**TH** (N=5)
Vitamin E (µg/dl)	12.4 ± 3.1	10.8 ± 3.5	10.5 ± 2.0	10.8 ± 2.8	11.2 ± 1.4

All values are mean ± SD.

NC=Control normal mice; NR=Normal mice receiving high dose; TC=Control thalassemic mice; TL=Thalassemic mice receiving low dose and TH=Thalassemic mice receiving high dose

4.2.2 Biochemical assessment

Parameters for evaluating biochemical assessment in this study were as follow:

4.2.2.1 Lipid profiles compose of levels of total cholesterol, triglyceride, free cholesterol and cholesteryl esters. Levels of total cholesterol, triglyceride, free cholesterol and cholesteryl esters in plasma from mice were shown in **Table 4.8**.

The level of total cholesterol of β-Thalassemic mice (TC) was lower than the group of normal mice (NC) with statistically significant ($P<0.05$). After receiving Riceberry bran oil in normal mice, total cholesterol of NR group mice increased compared to NC group. Similar results were found in TL and TH of β-Thalassemic mice compared to TC group. Furthermore, the level of total cholesterol in TL group was increased nearly to the level of total cholesterol in NC group.

The level of triglyceride in TC group was higher than NC group. After receiving Riceberry bran oil, the level of triglyceride in β-Thalassemic mice (TL and TH groups) has been statistically significant decreasing ($P<0.05$) when compared to TC group. This represented that rice bran oil had an effect in reducing the level of triglyceride in the group of β-Thalassemic mice. Moreover, the level of triglyceride in TH group also decreased with statistically significant when compared to TL group, while increased level was found in normal mice receiving rice bran oil (NR) compared to NC group.

Cholesteryl esters (CEs) which chromatogram has shown in this study were free cholesterol, cholesteryl arachidonate (CA), and cholesteryl linoleate (CL)

Free cholesterol in TC group was higher than NC group. After receiving Riceberry bran oil, free cholesterol in β-Thalassemic mice in TL group was slightly decreased compared to TC group. Oppositely, normal mice after receiving Riceberry bran oil (NR) free cholesterol increased compared to NC group.

Cholesteryl arachidonate (CA) in TC group was higher than NC group with statistically significant ($P<0.01$). After receiving Riceberry bran oil, β-Thalassemic mice in TL group had lower level of cholesteryl arachidonate while TH group had higher level when compared to TC group. Moreover, in normal mice

receiving Riceberry bran oil (NR) had increasing level of cholesteryl arachidonate compared to NC group with statistically significant (P<0.01).

No significant difference of Cholesteryl linoleate (CL) level had been found within a group of normal mice, β-thalassemic mice and among groups of normal and β-thalassemic mice.

Table 4.8 Levels of total cholesterol, free cholesterol and cholesteryl esters in plasma from mice

Lipid	**Levels in plasma, mmol/L**				
	NC	**NR**	**TC**	**TL**	**TH**
TC	2.14 ± 0.13 (5)	2.18 ± 0.20 (5)	1.69 ± 0.13 [aa](4)	2.02 ± 0.21 (3)	1.82 ± 0.25(3)
TG	0.63 ± 0.12 (5)	0.71 ± 0.20 (5)	0.73 ± 0.13 (4)	0.55 ± 0.03 [b] (3)	0.43 ± 0.08 [b, c](3)
FC	0.14 ± 0.04 (9)	0.18 ± 0.06 (9)	0.19 ± 0.01 (3)	0.18 ± 0.06 (3)	-
CA (20:4)	0.46 ± 0.10 (9)	0.61±0.11[aa](9)	0.74 ± 0.12 [a] (6)	0.72 ± 0.08 [a] (7)	0.75 ± 0.16 [a] (6)
CL (18:2)	0.22 ± 0.08 (9)	0.27 ± 0.10 (9)	0.32 ± 0.07 (6)	0.31 ± 0.07 (7)	0.34 ± 0.11 (6)

All values are mean ± SD. Number in parenthesis=number of mice in each group

[a] Significantly different from NC group: P<0.01, [aa] Significantly different from NC group: P<0.05,

[b] Significantly different from TC group: P<0.05, [c] Significantly different from TL group: P<0.05,

[d] Significantly different from NR group: P<0.05.

TC= total cholesterol; TG= triglyceride; FC= free cholesterol; CA= cholesteryl arachidonate; CL= cholesteryl linoleate. "-" = no data. NC=Control normal mice; NR=Normal mice receiving high dose; TC=Control thalassemic mice; TL=Thalassemic mice receiving low dose and TH=Thalassemic mice receiving high dose.

4.2.2.2 Liver and renal profiles compose of Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALK), Total protein, Albumin, Globulin, Total bilirubin and Uric acid.

Table 4.9 showed the results of liver and renal profiles which demonstrated that the level of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphotase (ALK), total protein, albumin, globulin, total bilirubin, and uric acid were all had no any significantly difference within normal mice, β-thalassemic mice and among normal and β-thalassemic mice.

Table 4.9 Liver and renal profiles in mice serum

Groups / Parameters	Normal Mice		β-Thalassemic mice		
	NC (N=5)	**NR** (N=5)	**TC** (N=4)	**TL** (N=3)	**TH** (N=3)
AST (U/L)	189 ± 175	234 ± 83	248 ± 84	254 ± 206	145 ± 45
ALT (U/L)	31.4 ± 10.6	35.0 ± 8.0	37.8 ± 10.4	40.0 ± 26.0	28.0 ± 7.8
ALK (U/L)	67.8 ± 13.2	70.6 ± 15.2	85.0 ± 13.8	78.0 ± 20.1	94.7 ± 8.3
Total protein (g/dL)	4.8 ± 0.2	4.6 ± 0.2	4.6 ± 0.1	4.7 ± 0.2	4.7 ± 0.2
Albumin (g/dL)	3.1 ± 0.1	2.9 ± 0.2	3.1 ± 0.1	3.1 ± 0.2	3.1 ± 0.1
Globulin(g/dL)	1.7 ± 0.2	1.7 ± 0.1	1.5 ± 0.1	1.6 ± 0.2	1.6 ± 0.2
Total bilirubin (mg/dL)	0.12 ± 0.04	0.10 ± 0.00	0.18 ± 0.05	0.17 ± 0.06	0.20 ± 0.10
Uric acid (mg/dL)	2.4 ± 0.5	3.5 ± 1.5	1.4 ± 0.3 [a]	1.8 ± 0.4	1.4 ± 0.9

All values are mean ± SD.

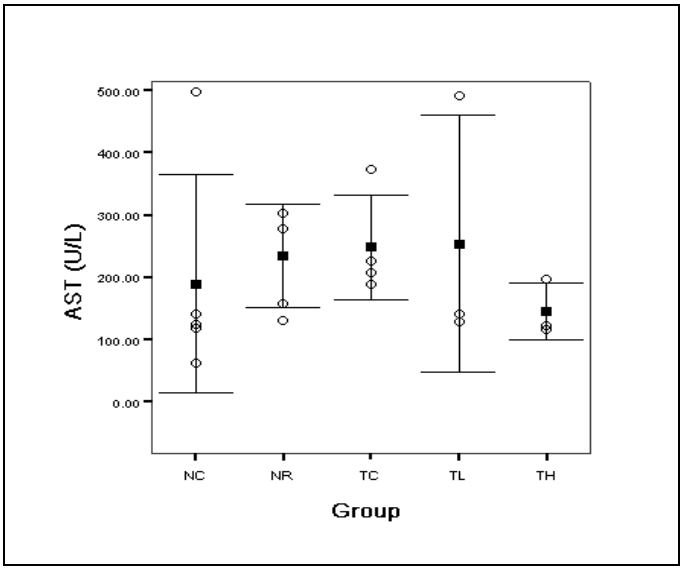
[a] Significantly different from NC group: $P<0.05$.

AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; ALK, Alkaline phosphatase.

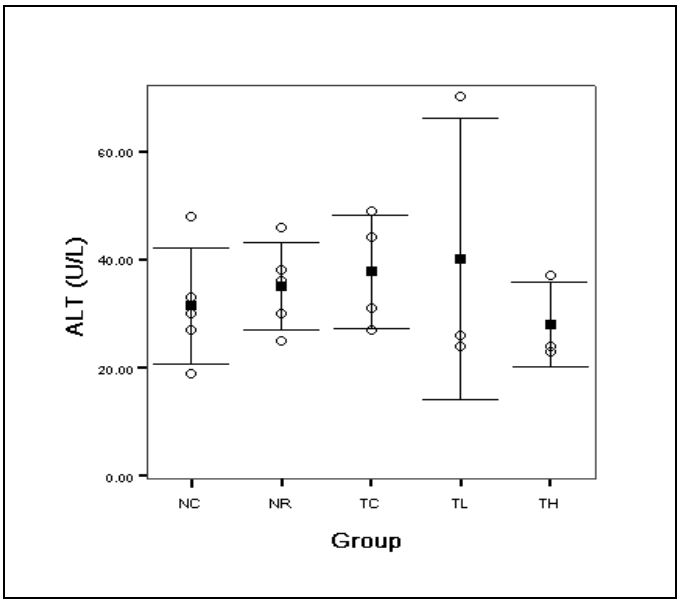
NC=Control normal mice; NR=Normal mice receiving high dose;
TC=Control thalassemic mice; TL=Thalassemic mice and TH=Thalassemic mice receiving high dose

Figure 4.2 Scatter plot and error bar of liver and renal profiles in mice serum

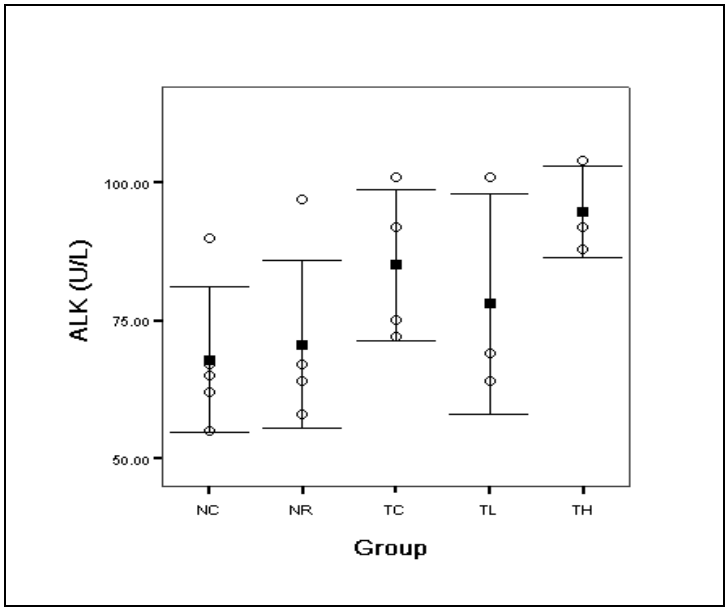
Aspartate aminotransferase (AST)



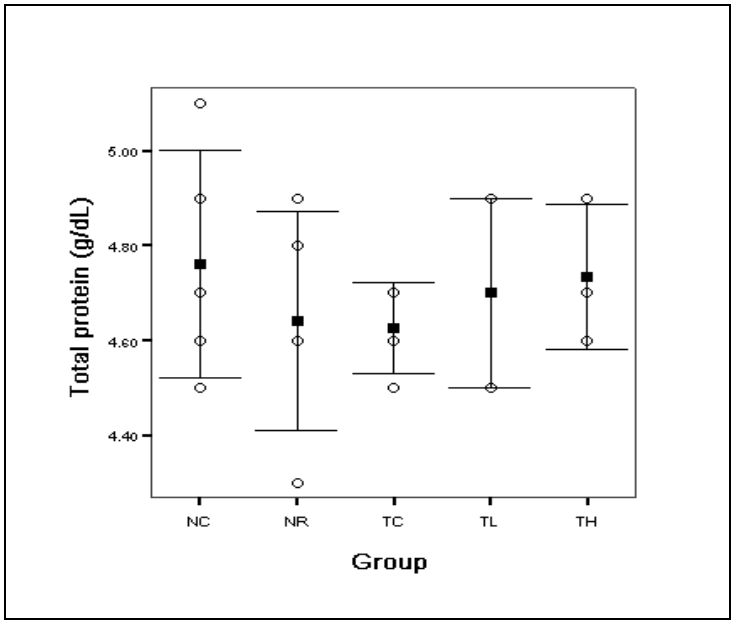
Alanine aminotransferase (ALT)



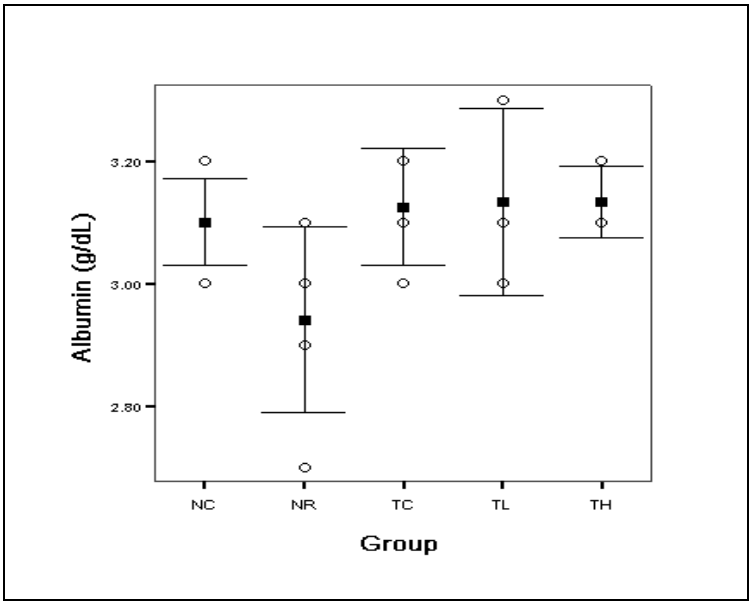
Alkaline phosphatase (ALK)



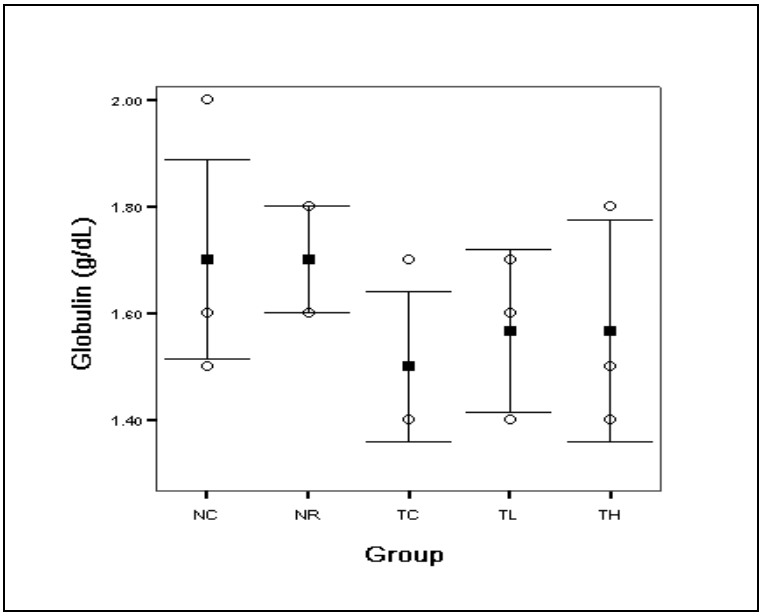
Total protein



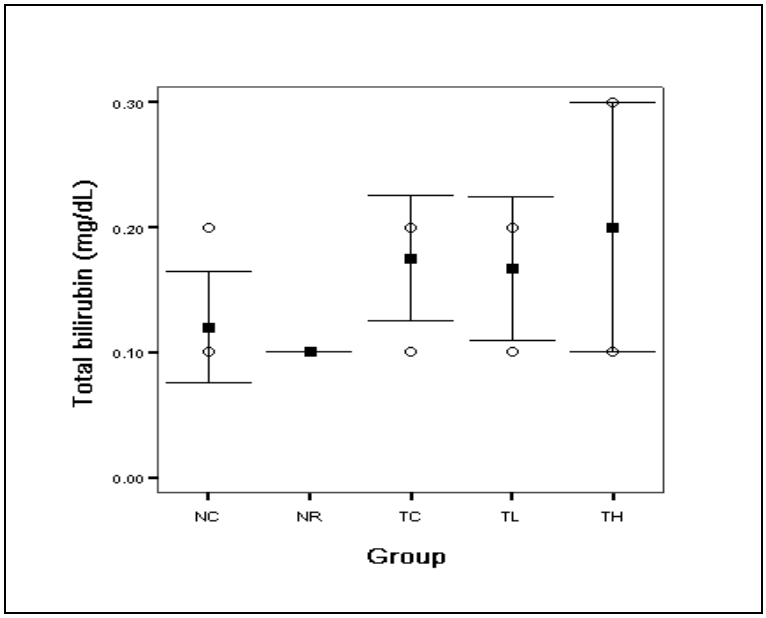
Albumin



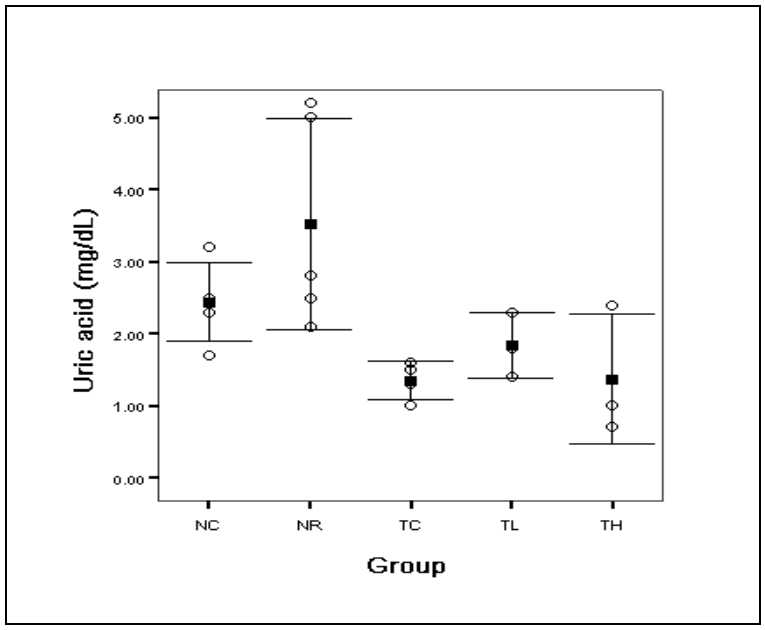
Globulin



Total bilirubin



Uric acid



NC=Control normal mice; NR=Normal mice receiving high dose;

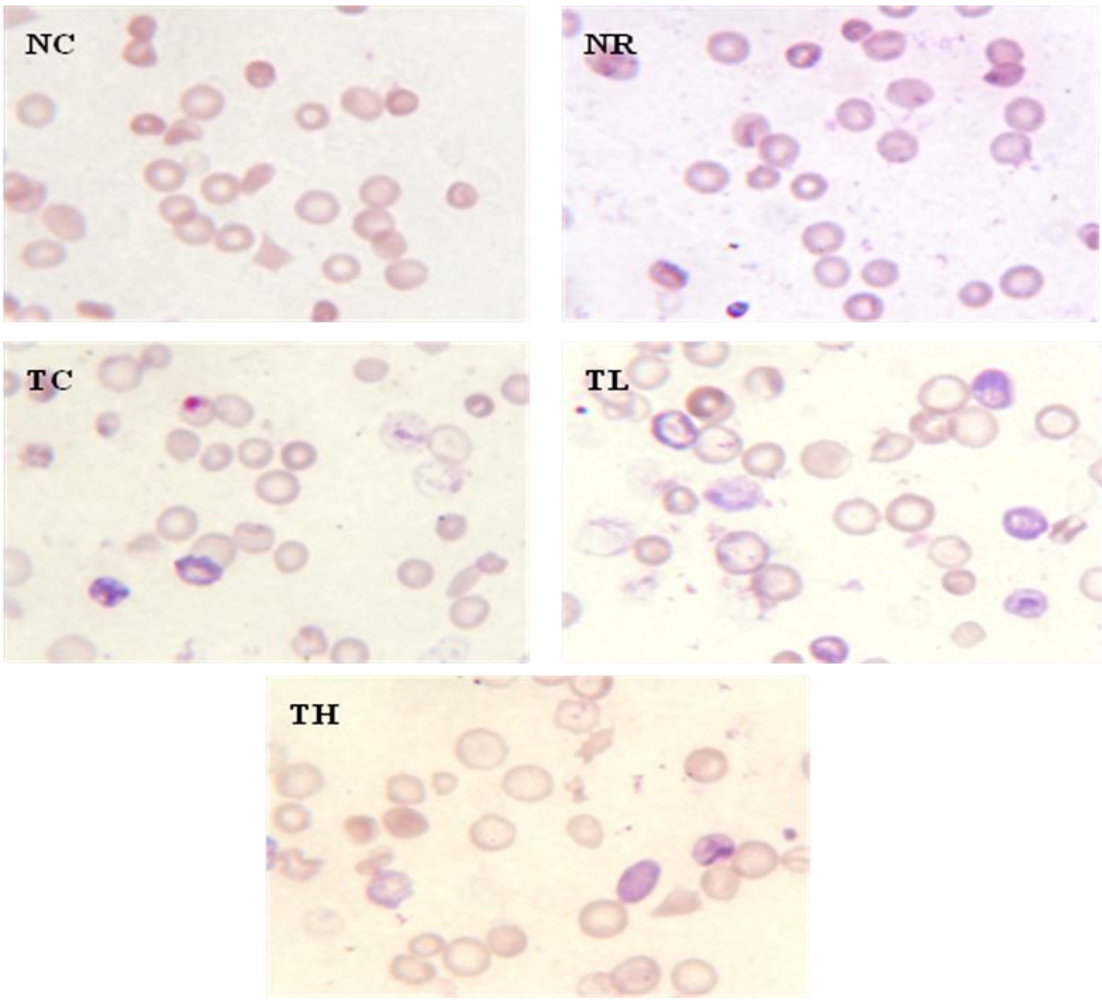
TC=Control thalassemic mice; TL=Thalassemic mice and TH=Thalassemic mice receiving high dose

4.2.3 Hematological assessment

4.2.3.1 Red blood cell morphology

Red blood cell morphology pictures of mice in different groups were shown in **Figure 4.3**. In normal mice, NR group had morphological blood pictures similar to those of NC group with normochromic, normocytic and red blood cells. In β-thalassemic mice, TC group had anisocytosis, frequent target cells, poikilocytosis with microcytes and polychromatic macrocytes. After receiving Riceberry bran oil in TL and TH groups, there was no change of red blood cell morphology compared with TC group.

Figure 4.3 Red blood cell morphology pictures of mice



NC=Control normal mice; NR=Normal mice receiving high dose; TC=Control thalassemic mice; TL=Thalassemic mice receiving low dose and TH=Thalassemic mice receiving high dose

4.2.4 Pathological assessment

4.2.4.1 Hepatic malondialdehyde

The result of hepatic malondialdehyde shown in **Table 4.10** demonstrates that the β-Thalassemic mice in TC, TL and TH groups had a significantly higher hepatic malondialdehyde ($P<0.01$) when compared to the normal mice (NC group). After receiving Riceberry bran oil, the difference in hepatic malondialdehyde among TC, TL and TH groups was not significantly different. Normal mice which receiving rice bran oil (NR) had higher level of hepatic malondialdehyde but without any statistically significant differences when compared to NC group.

Table 4.10 Hepatic malondialdehyde of mice

Groups / Parameter	Normal Mice		β-Thalassemic mice		
	NC (N=14)	**NR** (N=14)	**TC** (N=10)	**TL** (N=10)	**TH** (N=10)
Hepatic MDA (μmol/g dry wt.)	1.042 ± 0.221	1.149 ± 0.132	1.398 ± 0.143 [a]	1.365 ± 0.126 [a]	1.378 ± 0.109 [a]

All values are mean ± SD.

[a] Significantly different from NC group: $P<0.01$

NC=Control normal mice; NR=Normal mice receiving high dose; TC=Control thalassemic mice; TL=Thalassemic mice receiving low dose and TH=Thalassemic mice receiving high dose

4.2.4.2 Hepatic iron contents

The result of hepatic iron contents as shown in **Table 4.11** demonstrated that the β-Thalassemic mice in TC, TL and TH groups had a high hepatic iron contents when compared to the normal mice (NC group) with statistically significant (P<0.01). After receiving Riceberry bran oil, there was no statistical significant different in liver iron among TC, TL and TH groups. Normal mice which receiving rice bran oil (NR) had higher level of hepatic iron contents but without any statistically significant differences when compared to NC group.

Table 4.11 Hepatic iron content of mice

Groups / Parameter	Normal Mice		β-Thalassemic mice		
	NC (N=14)	**NR** (N=14)	**TC** (N=10)	**TL** (N=10)	**TH** (N=10)
Liver iron (mgFe/g dry wt.)	0.29 ± 0.10	0.33 ± 0.12	1.00 ± 0.32 [a]	1.15 ± 0.51 [a]	1.13 ± 0.53 [a]

All values are mean ± SD.

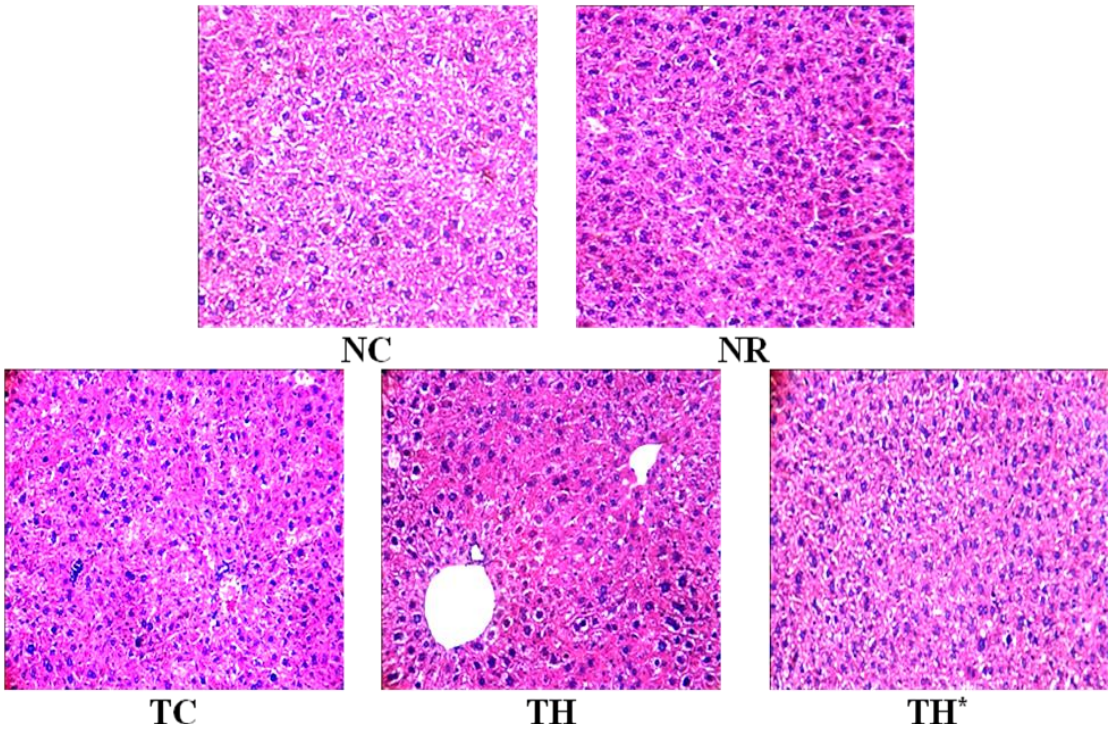
[a] Significantly different from NC group: P<0.01

NC=Control normal mice; NR=Normal mice receiving high dose; TC=Control thalassemic mice; TL=Thalassemic mice receiving low dose and TH=Thalassemic mice receiving high dose

4.2.4.3 Histopathologic studies of livers

Histopathologic studies of livers in different groups of mice were shown in **Figure 4.4**. General appearance of thalassemic livers was similar to those of the normal mice. However, β-thalassemic mice had higher extramedullary hematopoiesis and along the sinusoid-lining cells (Kupffer cells) contained finely granular brown pigment. After receiving Riceberry bran oil, the histology of livers in NR group was comparable to that of the NC group. Similarly, no significant change was observed in the livers of β-thalassemic mice after receiving Riceberry bran oil in the TH group, or even the group with extra high dose of Riceberry bran oil (72 mg/20body weight of mice) which was performed during the preliminary period (TH* group)

Figure 4.4 Histopathologic studies of livers in different groups of mice



NC=Control normal mice; NR=Normal mice receiving high dose; TC=Control thalassemic mice; TH=Thalassemic mice receiving high dose and TH*=double amount of high dose

4.2.4.4 Weight of organs

Each mouse had different body weight. To compare weight of organs of different body weight in this study, weight of organs were divided by body weight of each mouse. Weight of organs in this study such as liver, left-right kidneys, heart, lung, and spleen were shown in **Table 4.12** and **Figure 4.5**. The result showed that weight of liver, left-right kidneys and lung were not significantly difference within normal mice, β-thalassemic mice and among normal and β-thalassemic mice. While the weight of heart of β-Thalassemic mice in TC group was higher than that of normal mice in NC group with statistically significant at $P<0.01$. After receiving Riceberry bran oil, weight of heart in NR group when compared to NC group and TL and TH groups when compared to TC group did not changed. The weight of spleen, β-Thalassemic mice in TC group was higher than that of NC group with statistically significant at $P<0.01$. After receiving Riceberry bran oil, the weight of spleen in NR group when compared to NC group, TL and TH groups when compared to TC group did not changed. Moreover, weight of spleen in TL and TH groups were still higher than that of NC group with statistically significant at $P<0.01$and NR group with statistically significant at $P<0.01$.

Table 4.12 Weight of organs in mg/g body weight

Groups / Organ weight (mg/g body wt)	Normal mice		β-Thalassemic mice		
	NC (N=14)	**NR** (N=14)	**TC** (N=10)	**TL** (N=10)	**TH** (N=10)
Liver	52.65 ± 5.49	50.76 ± 4.53	54.29 ± 4.89	55.97 ± 3.29	54.99 ± 4.72
Kidney (Lt)	6.29 ± 0.53	6.69 ± 0.58	6.30 ± 0.41	6.42 ± 0.62	6.39 ± 1.16
Kidney (Rt)	6.21 ± 0.96	6.21 ± 0.76	6.00 ± 0.41	6.05 ± 0.72	6.13 ± 0.82
Heart	4.89 ± 0.57	5.04 ± 0.58	6.35 ± 0.62 [a, b]	6.21 ± 0.81	6.17 ± 1.28
Lung	6.80 ± 1.04	6.82 ± 0.94	7.12 ± 1.19	7.13 ± 0.74	7.92 ± 1.52
Spleen	3.22 ± 0.59	3.15 ± 0.70	15.61 ± 3.07 [a, b]	12.29 ± 3.63 [a, b]	15.40 ± 5.20 [a, b]

All values are mean ± SD.

Significantly different from NC group: [a] $P<0.01$

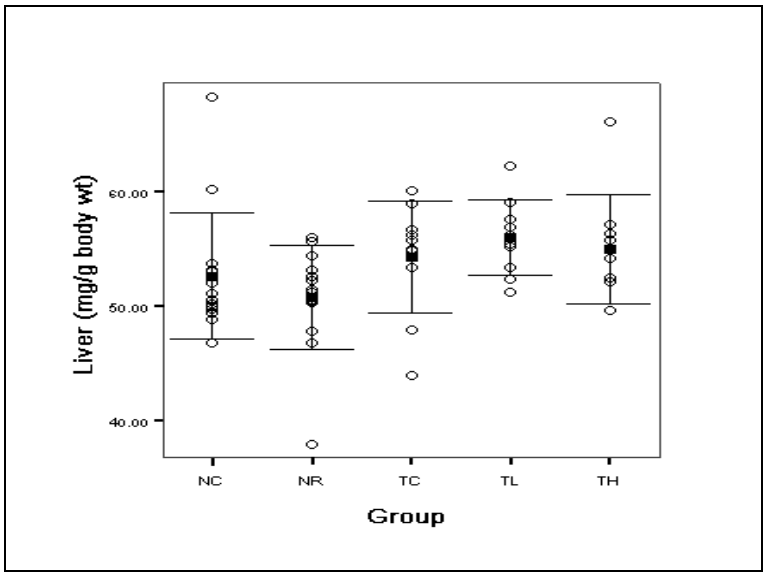
Significantly different from NR group: [b] $P<0.01$

NC=Control normal mice; NR=Normal mice receiving high dose; TC=Control thalassemic mice;

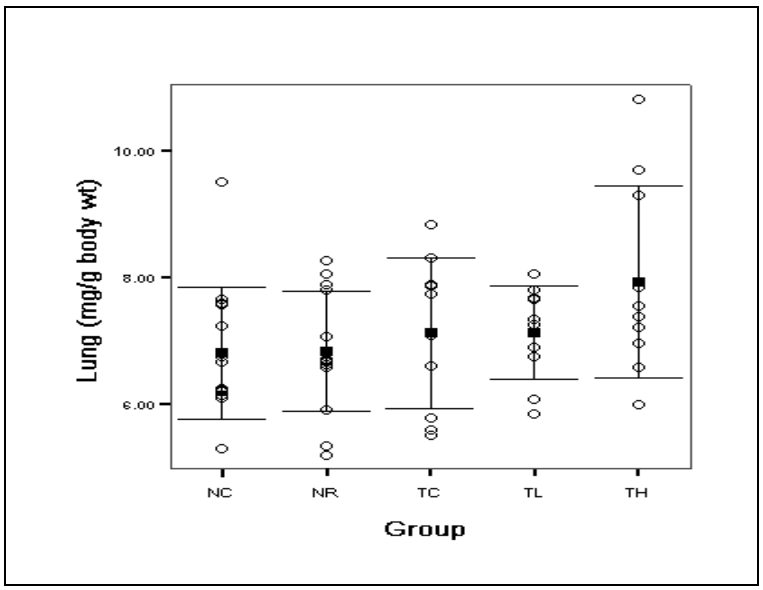
TL=Thalassemic mice receiving low dose and TH=Thalassemic mice receiving high dose

Figure 4.5 Weight of organs in mg/g body weight

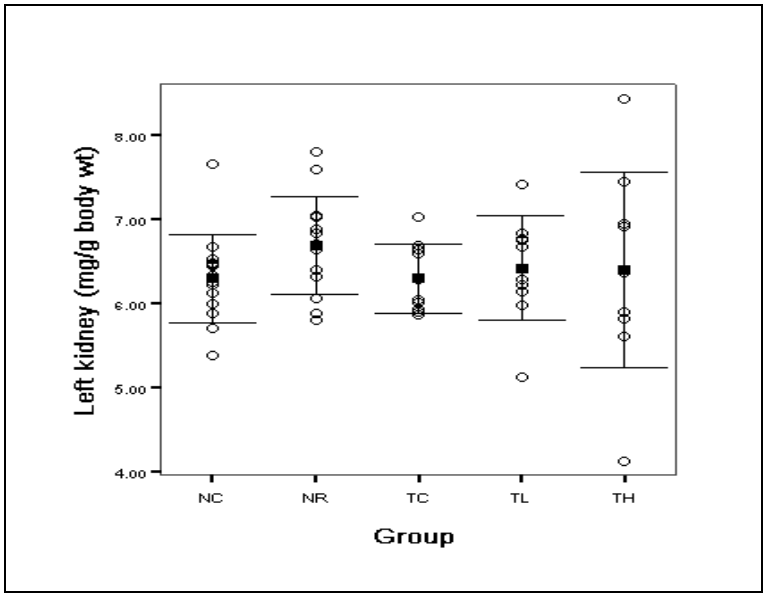
Liver



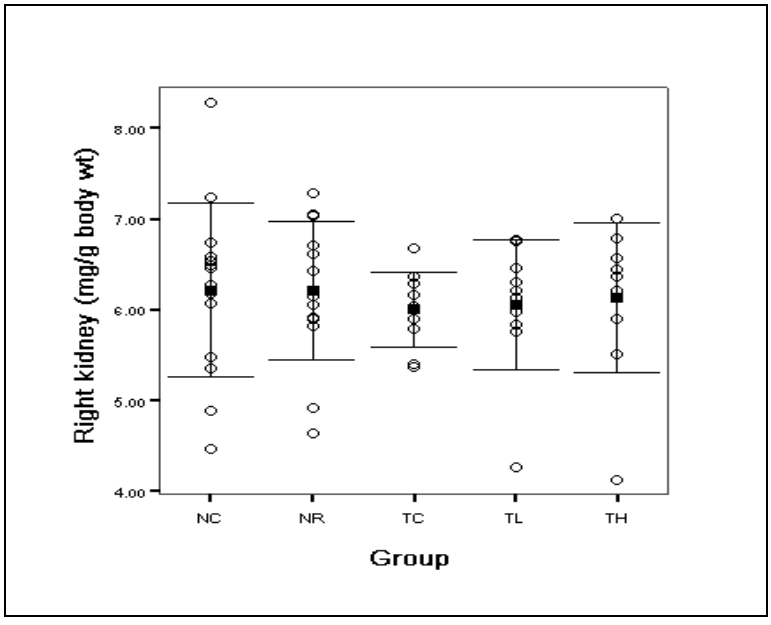
Lung



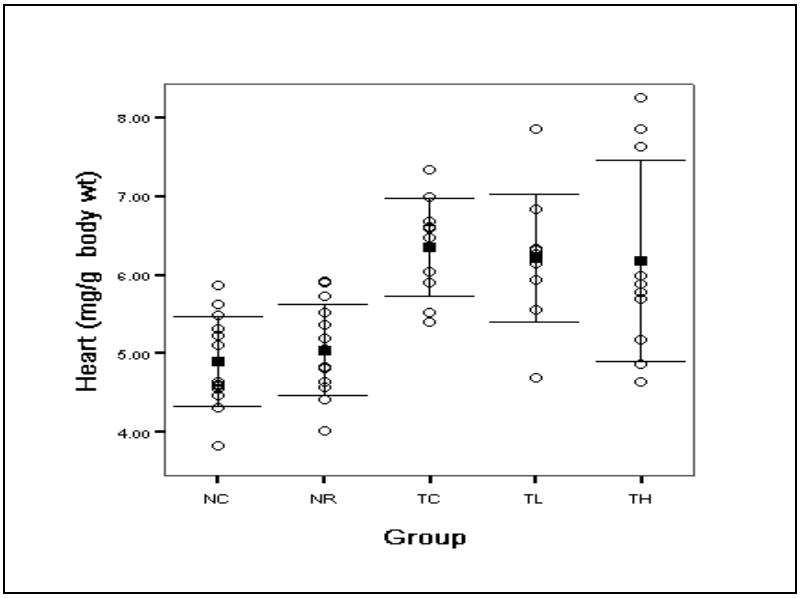
Left kidney



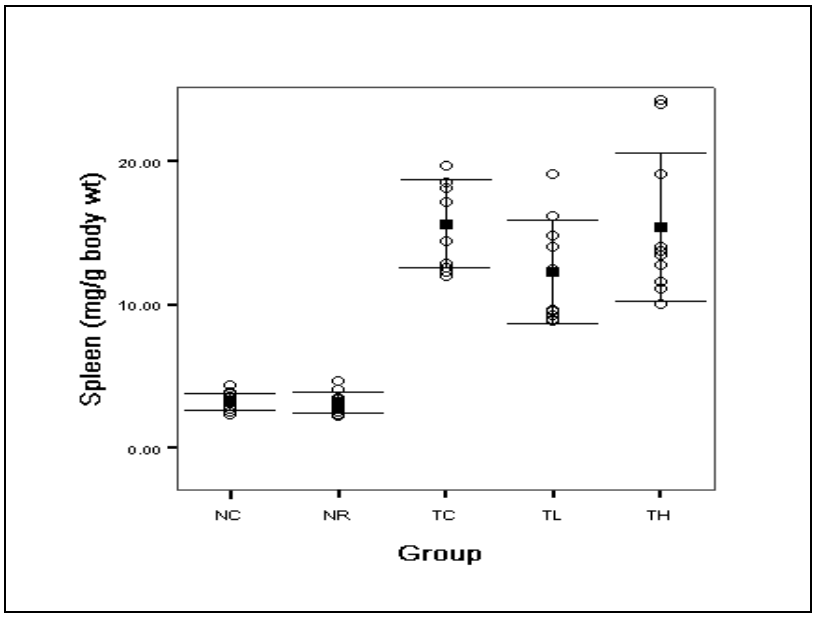
Right kidney



Heart



Spleen



NC=Control normal mice; NR=Normal mice receiving high dose;

TC=Control thalassemic mice; TL=Thalassemic mice and TH=Thalassemic mice receiving high dose

4.3 The correlation between oxidative stress status, antioxidant, biochemical, and pathological parameters

Before receiving Riceberry bran oil, the correlations of hepatic iron content and other parameters are shown in **Figure 4.6-4.9**. It was found that Vit E (p<0.05, r = -0.53), CoQ_{10} (p<0.01, r = -0.76) had negative correlation with hepatic iron whereas positive correlation was found with Reactive oxygen species (ROS) (p<0.05, r = 0.70) and hepatic malondialdehyde (p<0.01, r = 0.70). After receiving Riceberry bran oil, there was no correlation.

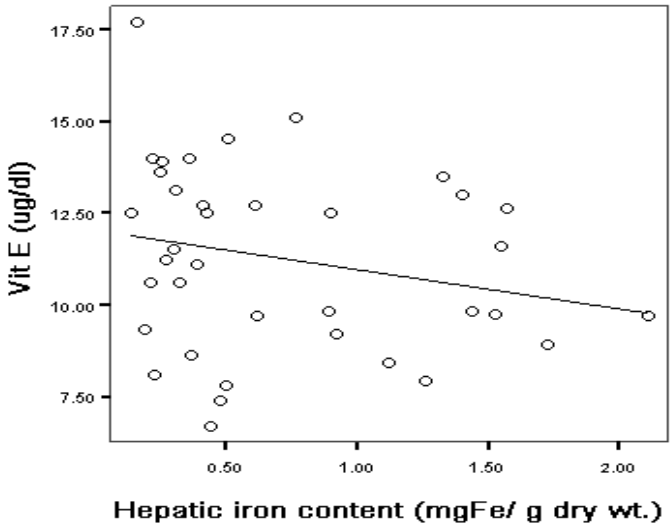


Figure 4.6 Correlation between Vit E and Hepatic iron content

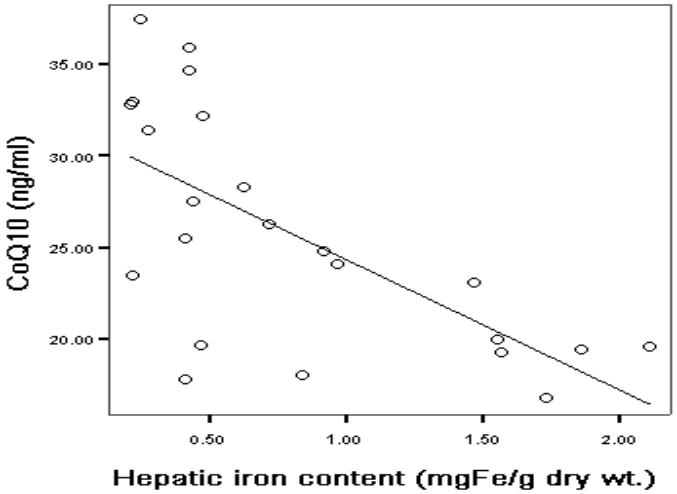


Figure 4.7 Correlation between CoQ_{10} and Hepatic iron content

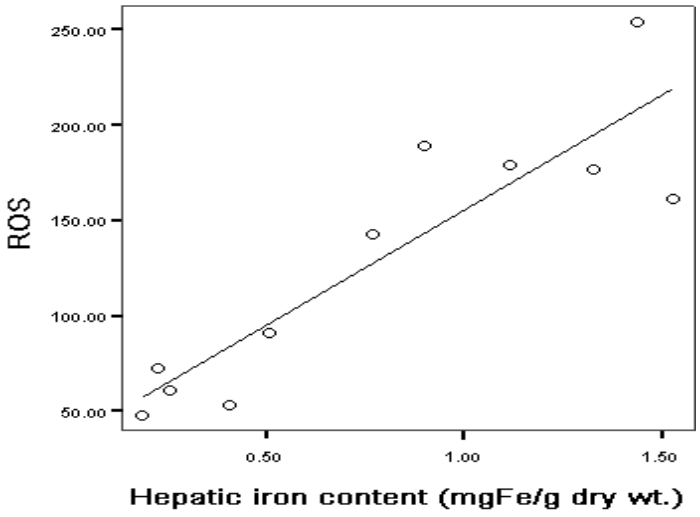


Figure 4.8 Correlation between Reactive oxygen species and Hepatic iron content

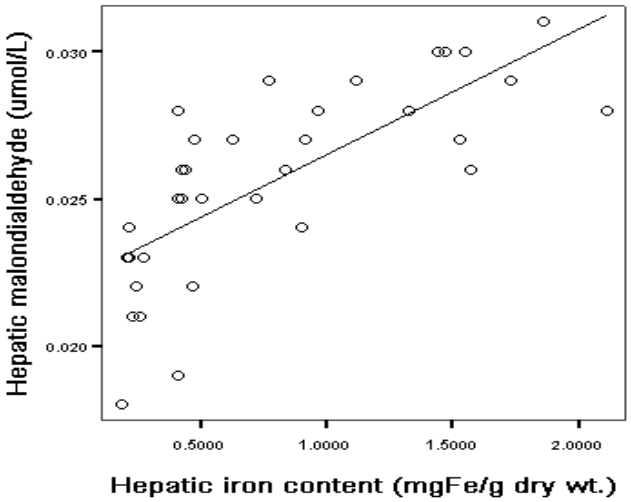


Figure 4.9 Correlation between hepatic malondialdehyde and Hepatic iron content

4.4 Riceberry bran oil profiles during a period of study

Riceberry bran oil profiles during a period of study were shown in **Table 4.13**. The result presented that since beginning (April 2009) to at the end of the study (December 2009), verify antioxidant and antioxidant contents of Riceberry bran oil did not changed so much such as Gamma-oryzanol ranged from 12.9 to 14.1 mg/g, Beta carotene 3.96 to 4.34 µg/g, lutein 12.0 to 20.4 µg/g, Vitamin E 0.97 to 0.99 mg/g, Polyphenol 0.42 to 0.46 mg/g, CoQ_{10} 23.1 to 24.8 µg/g, and ORAC ranged from 183 to 206 µmoles Trolox/g. The iron content of the studied Riceberry bran oil was 0.0012 mg/ g.

Table 4.13 Riceberry bran oil profiles during a period of study

Profile / **Time**	**Gamma-oryzanol** (mg/g)	**Beta carotene** (µg/g)	**Lutein** (µg/g)	**Vit E** (mg/g)	**Polyphenol** (mg/g)	**CoQ_{10}** (µg/g)	**ORAC** (µmoles Trolox/g)
1st sampling	13.7	4.06	19.3	0.98	0.43	24.2	206
	12.9	4.18	20.4	0.98	0.42	24.8	183
2nd sampling	14.1	3.96	12.0	0.99	0.44	23.1	206
	14.1	4.34	12.1	0.97	0.46	23.4	196
Mean±SD	13.7 ± 0.6	4.14 ± 0.16	15.9 ± 4.5	0.98 ± 0.01	0.44 ± 0.02	23.9 ± 0.8	198 ± 11

CHAPTER V
DISCUSSION

5.1 Oxidative stress status and antioxidants

Reactive oxygen species (ROS) in control group showed that β-thalassemic mice (TC) had the ROS level higher than that of the normal mice (NC) with statistical significance which reflected a highly oxidative stress in red blood cell of β-thalassemic mice. This result confirmed the previous studies in β-thalassemic mice and β-thalassemia patients (112,122). Wannasuphaphol *et al*. (122) reported that the level of ROS in β-thalassemic mice of this phenotypes (β-knockout) was higher than in the group of normal mice (wild type) and the other phenotypes of β-thalassemic mice such as Hb E-trangenic heterozygote (HbE-Tg heterozygote) and HbE-double heterozygote (HbE-DH) with statistical significance (p<0.0001) (28). The data from β-thalassemia patients showed that both free α-globin chains and free iron induce the generation of reactive oxygen species in their red blood cell (112). The level of hepatic iron in all groups were significantly correlated very well with ROS (p<0.01, r =0.82). This explained the increase in oxidative stress when the liver accumulated more iron.

After receiving Riceberry bran oil: in normal mice with high dose, NR group, the ROS level decreased compared to the NC group. In β-thalassemic mice, TL group had ROS level lower than that of TC group, while higher ROS was found in TH group. This probably because of Riceberry bran oil has an effect on β-thalassemic mice in performing itself as antioxidant in a certain level. When the dose of Riceberry bran oil is exceeded the optimum level, it may cause of the ROS generation. While in normal mice, Riceberry bran oil may be conductive to cause the reduction of ROS level even it received in high level equal to the dose that TH group of β-thalassemic mice has received, but still not much enough to generate ROS because the baseline level of ROS in normal mice was lower than in the β-thalassemic mice.

Lipid fluidity at the different regions in red blood cell membrane were assessed. The lipid fluidity near the area of phospholipids head group had no statistical

significant differences among these groups. The lipid fluidity in the area of hydrophobic region of red blood cell membrane in control group of β-thalassemic mice (TC), however, was lower than that of normal mice (NC) with statistical significance. This reflects the occurrence of oxidative stress which led to cross leakage of double bond in hydrophobic region of red blood cells in β-thalassemic mice. The alteration to decreased lipid fluidity cross leakage of double bond may come from oxidative damage to double bond in making change in the structure of lipid in the compound of red blood cell membrane which may cause changes in rotation of acyl chain as well as lipid fluidity leads to red cell membrane damage (123).

After receiving Riceberry bran oil: in β-thalassemic mice, TL and TH group had improved membrane fluidity closed to NC group. Lipid fluidity in TL group decreased with statistical significance compared to TC group. This demonstrated that low dose of Riceberry bran oil improved lipid fluidity in the area of hydrophobic region of red blood cell membrane in the β-thalassemic mice which may help to enhance strength of cell membrane and prolong red blood cell survival. While in normal mice, lipid fluidity in NR group did not change. Morales *et al.* (113) studied lipid fluidity in lipoprotein of thalassemia patients compared to healthy volunteers found that in thalassemia patients, there were an increasing of lipid fluidity in the area of hydrophobic region obviously, while there was a little change near the area of phospholipid head group of red blood cell membrane. Therefore in the area of hydrophobic region of lipoprotein of thalassemia patients is the target position of generate oxidative damage.

Malondialdehyde (MDA) which is the product of lipid peroxidation showed the lower level in control β-thalassemic mice (TC) compared to normal mice (NC) but no statistical significant difference. This finding was contrary to the result of previous studies in β-thalassemia patients whose plasma MDA was higher than normal subjects with statistical significance (124-125).

After receiving Riceberry bran oil: in normal mice, NR group, the MDA level decreased 7% compared to the NC group. In β-thalassemic mice, TL and TH groups, the MDA level decreased 15% and 31% respectively compared to the TC group but without any statistical significant difference. This demonstrated that Riceberry bran oil probably help both normal mice and β-thalassemic mice to decrease

lipid peroxidation as indicated by MDA parameter. This is confirmed by the correlation of MDA/total cholesterol and ROS ($p<0.01$, r =-0.79).

Superoxide dismutase (SOD) is an enzymatic antioxidant. It was found that SOD of β-thalassemic mice (TC) was higher than that of normal mice (NC). According to Kassab-Chekir *et al*. (126), SOD was found to be increased in β-thalassemia patients when compared to normal subjects. An increase in SOD reflects the presence of a severe oxidative stress situation in β-thalassemia patients.

After receiving Riceberry bran oil: in normal mice, the oxidative stress decreased as shown by the lower level of SOD compared to the normal mice with no Riceberry bran oil supplement. High dose of Riceberry bran oil showed the effect on decreasing in SOD of β-thalassemic mice.

Riceberry bran oil used in this study had antioxidant CoQ_{10} which was different from general rice bran oils (18). The benefit of CoQ_{10} in Riceberry bran oil was assessed in normal and thalassemic mice. The result showed that in control group: β-Thalassemic mice (TC) had CoQ_{10} level lower than in normal mice (NC) with statistical significance which agree with the study of Kalpravith R *et al*. (127) and Zita *et al.* (128) which found that the level of CoQ_{10} was very low in thalassemia patients compared to normal subjects.

After receiving Riceberry bran oil**,** the result showed an effect on the level of CoQ_{10} in both normal mice and β-thalassemic mice. In normal mice, the level of CoQ_{10} in NR group was significantly increased compared to the NC group. In β-thalassemic mice, the level of CoQ_{10} had been increased both TL and TH groups when compared to TC group which statistically significant in the TH group. Moreover, CoQ_{10} which has property to recycle antioxidant vitamin E (α-tocopherol) after being damaged by free radical, (129) did not demonstrate any correlation between CoQ_{10} and vitamin E levels in this study.

Vitamin E has function as a chain-breaking antioxidant that prevent the propagation of the reaction of free radical (130). This study found that in control group of β- thalassemic mice (TC), the vitamin E levels was lower than that of normal mice (NC). Similar to the previous study that low plasma or serum vitamin E level in thalassemia patients were found compared to normal subjects (10).

After receiving Riceberry bran oil, vitamin E levels in normal mice (NR) was decreased while vitamin E levels in β-thalassemic mice (TL and TH) were increased but without any significant differences. This demonstrated that Riceberry bran oil increase vitamin E levels in β-thalassemic mice with the dose dependent. Vitamin E content from two doses of Riceberry bran oil were 46 and 100 mg/day of low and high dose, respectively, which consider to be lower than the previous report for treatment of thalassemia patients (10). Suthutvoravut *et al.* (10) found that after vitamin E supplementation 200 mg/day for 4-6 weeks to thalassemia patients, their vitamin E levels increased to normal value. Pfeifer *et al*. (131) reported that after vitamin E supplementation 400 IU/day (267 mg) for 3 months to β-thalassemia intermedia patients, ROS levels of red blood cells and MDA has decreased significantly when compared to before supplementation and to healthy subjects. However, there was a study of Das *et al*. (124) in β-thal/Hb E thalassemia patients, β-thalassemia patients, and normal subjects found that initially, there was not much difference in plasma vitamin E levels in all group. After vitamin E therapy 10 mg/kg/day for 4 weeks, vitamin E levels in plasma and red blood cells of β-thal/Hb E thalassemia patients has increased significantly when compared to untreated β-thal/Hb E thalassemia while β-thalassemia patients did not show any improvement. However, lipid peroxidation has remarkably decreased as well as SOD activity that has decreased to normal value both in β-thal/Hb E thalassemia patients and β-thalassemia patients after treated with vitamin E.

5.2 Biochemical assessment

Rice bran oil contains high level of monounsaturated fatty acid (MUFA) (135). This study found that Riceberry bran oil was also contained high level of MUFA by having percentage ratio of: Saturated fatty acid (SFA): Monounsaturated fatty acid (MUFA): Polyunsaturated fatty acid (PUFA) = 17:30:22. Although the mechanism of action of rice bran oil on lipid metabolism is still not certainly known. However, there were many studies demonstrated that rice bran oil has property to improve the plasma lipid pattern of rodents, rabbit, non-human primates, and human (136-140). Rhaghuram *et al*. and Sugano *et al*. (141-142) found that rice bran oil had

ability to decrease total cholesterol (TC) and LDL-cholesterol (LDL-C) by about 40% and increase high density lipoprotein-cholesterol (HDL-C) level. While Cieco *et al*. (143) found that rice bran oil had ability to decrease TC, LDL-C and TG by about 20% but did not significantly increase HDL-C level.

However, rice bran oil is also containing of specific contents of phytosterols and vitamin E. Phytosterols are compounds which occur naturally and similar to cholesterol structure but have different side-chain configurations. Phytosterol are present in rice bran oil such as gamma-oryzanol. Gamma-oryzanol is the compound which is occurring naturally only in rice bran (144). According to the previous study, in difference animal models which found that rice bran oil and unsaponifiable cause significantly increased the fecal excretion of acid and neural sterols and that result may be come from gamma-oryzanol (63). When feed gamma-oryzanol 0.5% with high cholesterol diet in male albino rats, the finding showed that bile flow and total bile acid output have been increasing 12% and 18% respectively as well as fecal excretion of cholesterol and bile acid that have been increasing significantly 28% and 29% respectively, while cholesterol absorption is decreasing 20%. In control group there was no changes in bile flow and composition (145). Gamma-oryzanol may act in 3 ways: 1) by increasing of fecal bile acid excretion, 2) conversion of cholesterol to bile acids and 3) inhibit of cholesterol absorption. Moreover, gamma-oryzanol's property helps to reduce the risk of cardiovascular disease in animals (rats, hamsters and rabbits) (143). However, pharmacological mechanism is still not certainly known.

In addition, phytosterols that found in crude rice bran oil have 3 groups: 4.4'-dimethylsterols, 4-monomethyl-sternols, and 4-desmethylsterols (54). In some plant food and vegetable oils have shown that nuts and oils contain higher level of sterols ($\geq$ 1%) than in fruits and vegetables (<0.05%). Rice bran oil has a high level of plant sterol as shown in Table 5.1(55).

Table 5.1 Some reported sterol concentrations in selected foods and vegetable oil (mg/ 100 g)

Food	Phytosterol
Potato	5
Tomato	7
Pear	8
Lettuce	10
Carrot	12
Apple	12
Onion	15
Banana	16
Fig	31
Garbanzo bean	35
Kidney bean	127
Soybean	161
Pecan	108
Almond	143
Cashew nut	158
Peanut	220
Sesame seed	714
Peanut oil	207
Olive oil	221
Soybean oil	250
Cottonseed oil	324
Safflower oil	444
Sesame oil	865
Corn oil	968
Rice bran oil	1190

Phytosterols are known to to inhibit oxidative deterioration of oil serving as potential antipolymerization agents for frying oils. According to the previous study found that rice bran oil is a kind of oil that is suitable to fry food because of its high smoke point. This property may be related to the relatively high content in 4-monomethyl-sterols with an ethylide side chain that may contribute to its oxidative stability (146-147). Moreover, phytosterol antihypercholesterolemic effect has been clearly observed in different studies on human subjects and offering cardiologic health benefits (148-149).

Vitamin E is classified into 2 main classes as tocopherol and tocotrienol which found that 70% of vitamin E in rice bran oil is tocotrienols, which may be the main mediators of rice bran oil that have an antihypercholesterolemic effect (141, 143). Their hypocholesterolemic activity has been clearly demonstrated in different animal species (150-151) and human (152-153) which found that it can inhibit 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCoA-R), a key enzyme in endogenous synthesis of cholesterol through two post-transcription actions, increase the controlled degradation of reduce protein and reduce the efficiency of translation of

HMGCoA-R messenger RNA (154-155). Riceberry bran oil in this study had the level of tocopherol = 44-45% while tocotrienol = 55-56% of total vitamin E in Riceberry bran oil.

Many studies found that β-thalassemia patients had abnormal plasma lipid profiles, including low level of total cholesterol (TC), low level of high density lipoprotein cholesterol (HDL-C) and high level of plasma triglyceride (TG) when compared to normal subjects (132-134). In this study was conform to studies as mentioned above that the level of total cholesterol of β-thalassemic mice (TC) was lower than in normal mice (NC) with statistical significance. Moreover, the level of triglyceride in TC group was also high when compared to NC group.

Riceberry bran oil had no effect on the decreasing of total cholesterol level but increasing both in normal mice and β-thalassemic mice with no statistical significance. However, Riceberry bran oil improved triglyceride by statistically significant decreased the level in β-thalassemic mice (TL and TH) compared to TC group. Moreover, it was demonstrated that high dose of Riceberry bran oil had more effect on level of triglyceride than the low dose. In normal mice, Riceberry bran oil increase triglyceride in NR group when compared to NC group without any statistical significance. Moreover, this study did not correspond to the previous study (118) which found an abnormal lipoprotein profiles such as total cholesterol, free cholesterol, LDL-cholesterol, HDL-cholesterol, apolipoprotein A-1, apolipoprotein B, cholesteryl arachidonate and cholesteryl linoleate in plasma of thalassemia patients was lower than that of normal volunteers with statistical significance. Whereas the result from mice in this study showed that total cholesterol and triglyceride correspond to the above mentioned study but free cholesterol, cholesteryl arachidonate and cholesteryl linoleate in β-thalassemic mice (TC) were higher than that of normal mice (NC). Moreover, cholesteryl arachidonate in TC group was higher than in NC group with statistical significance.

Liver and renal profiles in mice serum found that aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALK), total protein, albumin, globulin, total bilirubin, and uric acid were not significantly different in all groups of animal.

This represented that Riceberry bran oil had no any effect on the change of liver measured by parameters such as AST, ALT, ALK, total protein, albumin globulin and total bilirubin and had no any effect on the change of kidney from parameter uric acid. In addition, the study of Yang *et al*. (27) found that the increasing of serum bilirubin in β-knockout mice indicated the increasing in red cell turn over and hemolysis while in this study found that β-thalassemic mice (TC) had higher total bilirubin than in normal mice (NC) but without any significant difference.

5.3 Hematological assessment

Red blood cell morphology of both β-thalassemic mice and normal mice in this study similar to the previous study (122) which found that red blood cell morphology of β-thalassemic mice (TC) found anisocytosis, frequent target cells, poikilocytosis with microcytes and polychromatic macrocytes. Such condition represented to the pathologic abnormal of red blood cells in β-thalassemic mice while normal mice (NC) found normochromic, normocytic which represented the normal of red blood cell morphology.

After receiving Riceberry bran oil, red blood cell morphology both in β-thalassemic mice (TL and TH) and normal mice (NR) showed no changes. This represented that Riceberry bran oil had no effect on the change of red blood cell morphology.

5.4 Pathological assessment

High hepatic iron contents and hepatic malondialdehyde found in the control group of β-thalassemic mice (TC) in this study supports the studies of Yang *et al.* (27) and Wannasuphaphol *et al*. (28) that β-knockout mice had the spontaneous iron decomposition which induces oxidation such as malondialdehyde. Hepatic iron contents as one of parameter to indicate the severity in β-thalassemia disease and also confirm iron deposition in liver. The increasing of hepatic iron contents indicated the progressive iron accumulation and also elevated red blood cells production and destruction.

After receiving Riceberry bran oil, in normal mice, NR group, showed a little increase in hepatic iron contents and hepatic malondialdehyde when compared to that of the NC group. In β-thalassemic mice, TL and TH groups were also changing just slightly with no statistical significant difference as well when compared to that of the TC group.

In addition, iron contents from two doses of Riceberry bran oil were found to be negligible (0.02 and 0.04 mg/day of low and high dose respectively). All mice were fed on the commercial diet which had no iron content. As the results, it would be concluded that Riceberry bran oil may not involved the iron deposit, oxidative stress as indicated by hepatic malondialdehyde in liver both normal mice and β-thalassemic mice.

When the correlation of hepatic iron contents and other parameters were assessed, it was found that Vit E ($p<0.05$, r = -0.53), CoQ_{10} ($p<0.01$, r = -0.76) had negative correlation with hepatic iron whereas that of positive correlation was found in Reactive oxygen species (ROS) ($p<0.05$, r = 0.70) and hepatic malondialdehyde ($p<0.01$, r = 0.70). These correlations indicated that iron induced oxidative stress whereas after receiving Riceberry bran oil, there was no correlation.

Histopathologic studies of livers of both β-thalassemic mice and normal mice in this study were similar to the previous study (122) in that the general appearance of thalassemic livers was similar to those of the normal mice. However, in β-thalassemic mice had higher extramedullary hematopoiesis and along the sinusoid-lining cells-Kupffer cells contained fine granular brown pigment. Such condition represented to the pathologic abnormal of liver in β-thalassemic mice while normal mice (NC) represented the normal of histopathologic studies of livers.

After receiving Riceberry bran oil, histopathologic studies of livers both in β- thalassemic mice (TL and TH) and normal mice (NR) showed no changes. This represented that Riceberry bran oil had no effect on the change of histopathologic studies of livers.

Weight of organs like left-right kidneys and lungs in β-thalassemic mice (TC) and normal mice (NC) were not different. The expansion of spleen's size about 5 times in β- thalassemic mice was to support appropriately highly increased removal of damage red blood cells and to provide a source of erythropoiesis. These result

supported the study of Yang *et al.* (27), Wannasupaphol *et al*. (28) and Jamsai *et al.* (156) that β-globin gene knockout mice developed splenomegaly. In addition, the weight of heart in TC group was higher than NC group with statistical significance; the heart size seemed to be increased in β-thalassemic mice indicating the overload function to maintain sufficient oxygen supply.

After receiving Riceberry bran oil, no change of organ's weight was observed in both in β-thalassemic mice and normal mice. This finding concluded that Riceberry bran oil had no effect on the change of organs' weight.

CHAPTER VI
CONCLUSION

1. Heterozygous β-globin gene knock-out mice is caused by the complete deletion of adult murine β-globin genes which lead to reduction or absence in β-globin synthesis and unmatched α-globin chain. These factors led to the increased oxidative stress as indicated by increased reactive oxygen species (ROS) in red blood cells, decreased lipid fluidity in the area of hydrophobic region of red blood cell membrane and high hepatic iron contents. While the level of antioxidant such as CoQ_{10} decreased. In addition, there was dyslipidemia showed low total plasma cholesterol. Splenomegaly and higher heart weight were found in all β-thalassemic mice when compared to normal mice in this study.
2. Gavaging Riceberry bran oil as a natural antioxidant were introduced with low dose (17 mg/day) in β-thalassemic mice (TL group) and high dose (36 mg/day) in both of normal and β-thalassemic mice (NR and TH group) for 2 months of study period.
3. For thalassemic mice, Riceberry bran oil tended to increase antioxidant levels such as vitamin E, CoQ_{10} and reduced oxidative stress shown by MDA and SOD especially for high dose group. For TL group, Riceberry bran oil decreased oxidative damage to double bond in the area of hydrophobic region (h_o/h_{-1}) of red blood cell membrane as indicated by decreased reactive oxygen species and significantly increased lipid fluidity. For TH group, Riceberry bran oil significantly increased the antioxidant level of CoQ_{10} about 25%. Decreased plasma triglyceride approximately 25 and 41% were demonstrated in TL and TH groups, respectively.
4. Although there was no significant difference among normal mice (NC and NR groups), the NR group showed less oxidative stress parameters such as ROS,

SOD and MDA than that of NC group. Riceberry bran oil also significantly increased the antioxidant level of CoQ_{10} approximately 24% in NR group.

5. Riceberry bran oil had no effect on free cholesterol, cholesteryl esters, liver and renal profiles, red blood cell morphology, hepatic malondialdehyde, hepatic iron content and organs.
6. Thalassemic mice fed with Riceberry bran oil showed reduced malondialdehyde and improved membrane fluidity and serum lipid profiles which could be resulted from antioxidant such as vitamin E and CoQ_{10}.
7. Riceberry bran oil had effect on antioxidants and oxidative stress status of normal and β-thalassemic mice but did not improve iron overload which was indicated by hepatic iron content.

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APPENDICES

APPENDIX A
DETERMINATION METHOD OF REACTIVE OXYGEN SPECIES (ROS) IN RED BLOOD CELLS

Principle

Reactive oxygen species (ROS) in red blood cells were measured by monitoring the formation of 2′, 7′-dichlorofluorescein (DCF), a highly fluorescent oxidation product which modified from Fibach's protocol (113). 2′, 7′ -dichlorofluorescein diacetate (DCFH-DA) crossed the cell membranes, subsequently undergoing deacetylation by intracellular esterases. The resulting of 2′, 7′-dichlorofluorescein (DCFH) was trapped inside the cells and was reacted with H_2O_2 to give the fluorescent 2′, 7′-dichlorofluorescein (DCF). The conversion of fluorescein showed in Figure 7.1 (156)

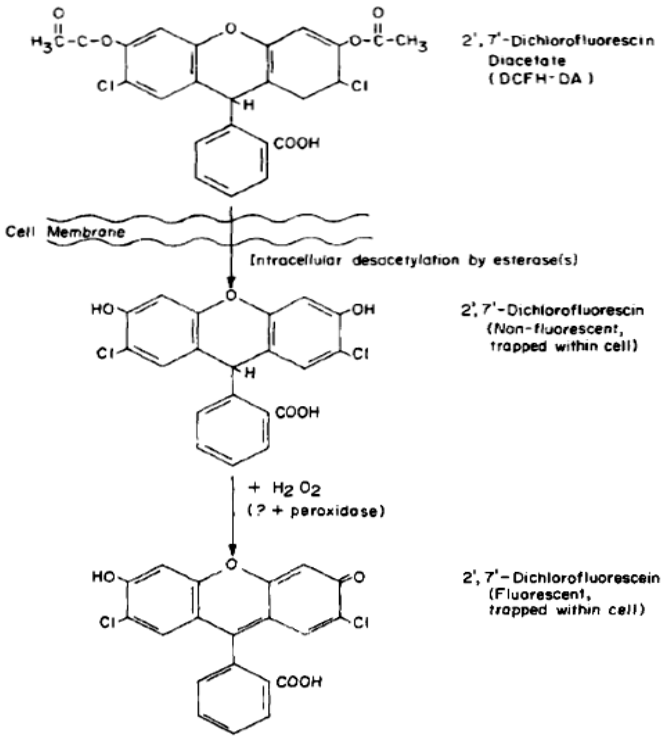


Figure 7.1 The conversion of 2′, 7′-dichlorofluorescein (DCF)

Reagents

1. 2´7´ Dichlorofluorescein diacetate (DCFH-DA) (Sigma Chemical Co., St. Louis, MO, USA
2. Methanol (MERCK, Darmstadt, Germany)
3. Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate buffer saline (PBS) GIBCO-Invitrogen, Grand Island, NY, USA)
4. 30% Hydrogen peroxide (Ajax Findchem, Aucklaand, New Zealand

Reagent preparation

1. 10 mM 2', 7'-dichlorofluorescein diacetate (DCFH-DA)
   - Weight DCFH-DA 5 mg dissolved in 1 ml methanol
2. 100 mM H_2O_2
   - Weight 11.4 μl of 30% H_2O_2 into 1 ml of PBS. This solution was prepared freshly.

Procedure

1. Diluted whole blood to a concentration of 1×10^6 RBCs/ ml with Ca^{2+}- and Mg^{2+}- free Dulbecco's phosphate buffer saline (PBS).
2. Pipette 2 ml of blood solution to a new 5-ml polypropylene tube.
3. Added 40 μl of DCFH-DA which dissolved in methanol to had final concentration 0.2 mM.
4. Incubation at 37 °c for 15 min in a humidified atmosphere of 5% CO_2 in air.
5. Added 2 ml of PBS
6. Centrifuged at 2,000 rpm for 5 min.
7. Removed supernatant and replaced cell with 1 ml PBS.
8. Mixed and aliquot into two tubes with equal amount
9. One tube of aliquot was oxidative stimulated by adding 5 μl of 100 mM H_2O_2.
10. The 1×10^6 RBCs/ ml of stimulated sample were studied by flow cytometer as well as unstimulated sample immediately.
11. Both samples were incubated for 20 min in the dark, at room temperature and analyzed again.

A two parameter dot-plot of the side light scatter (SSC) and forward light scatter (FSC) was primarily analyzed (Figure 7.2). Scattergram to obtain fluorescence intensity was interpreted between FL1 (DCF) and forward scatter (FSC). The mean fluorescence intensity of 5,000 intact red blood cells was analyzed at min 0 as control. Another 5,000 intact red blood cells at min 20 were quantified for reactive oxygen species level in mice.

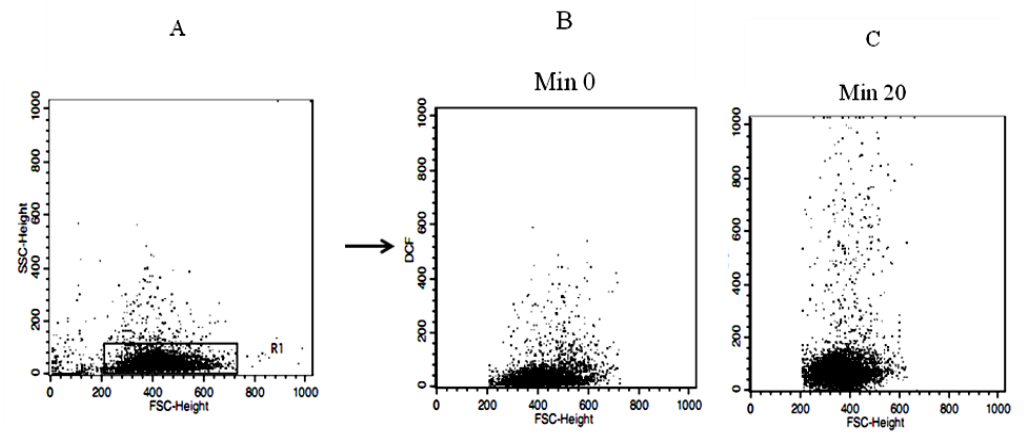


Figure 7.2 Analysis of DCF by Flow cytometry.
Red blood cells of mice were incubated with DCFH-DA for 20 min and stimulated by H_2O_2 to amplify fluorescence signal. The FSC versus SSC windows were demonstrated and gated to included only red blood cells in region R1 (Panel A) and then analyzed on DCF versus FSC dot plots to calculate the ROS level (Panel B-C).

APPENDIX B
DETERMINATION METHOD OF LIPID FLUIDITY OF RED BLOOD CELL BY ELECTRON SPINS RESONANCE (ESR) SPECTROSCOPY

Principle

Lipid fluidity of red blood cell membrane was measured by means of electron spin resonance (ESR) spectroscopy and spin labelling technique with two spin probe such as 5-doxyl stearic acid (5-DS) and 16-doxyl stearic acid (16-DS). 5-DS and 16-DS which had structure consisted of nitroxide stable radical molecules attached to molecules of stearic acid at carbon positions 5 and 16, respectively. When this spin probes molecules into plasma membrane, nitroxide stable radical was in the surface near the location of phospholipids polar group and location deep into the hydrophobic region of the red blood cell membrane. The rotation of nitroxide stable radical will correlate to lipid fluidity in that region.

Reagents

1. Hexane solution of 5-doxyl stearic acid (5-DS) and 16-doxyl stearic acid (16-DS) (Sigma,ST.Louis,MO)
2. Nitrogen
3. 0.9 % Normal Saline (0.9% NSS)

Procedure

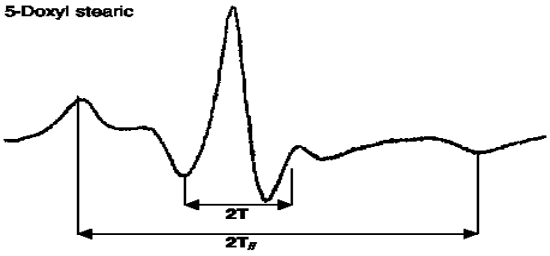
1. Take 50 µl of 0.1 mg/ml hexane solution of 5-DS and 16-DS dried under nitrogen in a clean glass tube.
2. Add 50 µl of red blood cells that washed with NSS to a concentration of 50% hematocrit and kept for 5 minutes at room temperature.
3. Transferred the labeled red blood cells to a capillary tube for measured ESR.

ESR spectrum were recorded by using X-band ESR (JEOL RX-2) spectrometer equipped with TE011 cavity. The microwave frequency was set at 9.4 GHz and at 5 mW, the amplitudes of 100 kHz field modulation at 0.25 and 0.125 mT for 5-DS and 16-DS respectively. The external magnetic field had rate of scanning at 5 mT/ min. ESR spectrum of 5-DS provided the order parameter (S) that presented the amplitude of motional anisotropy of the spin label nearby the polar head group of phospholipid layer which the order parameter can be calculated in according to the equation:

$$S = 1.732 \times (T_{//}\text{-}T\text{-}C) / (T_{//} + 2T + 2C); \quad C = 1.4\text{-}0.053(T_{//}\text{-}T)$$

while $T_{//}$ and T can be measured as showed in **Figure 7.3**.

Figure 7.3 X-band ESR of 5-doxyl stearic acid (5-DS) on red blood cell membrane



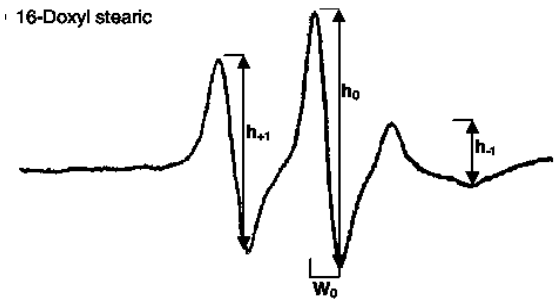
While 16-DS provided h_0/h_{-1} and the rotation correlation times could be measured as τ_B and τ_C which presented the spin label motion in the directions perpendicular in hydrocarbon of the deeper phospholipid layer. The rotation correlation times could be calculated in according to the following equation:

$$\tau_B = K\Delta W_0 [(h_0/h_{+1})^{1/2} - (h_0/h_{-1})^{1/2}],$$

$$\tau_C = K\Delta W_0 [(h_0/h_{+1})^{1/2} - (h_0/h_{-1})^{1/2}\text{-}2],$$

While h_{+1}, h_0, h_{-1} and W_0 could be measure as showed in **Figure 7.4**

Figure 7.4 X-band ESR of 16-doxyl stearic acid (16-DS) on red blood cell membrane



APPENDIX C
DETERMINATION METHOD OF MALONDIALDEHYDE (MDA) IN PLASMA AND HEPATIC MALONDIALDEHYDE

Principle

Malondialdehyde is a maker that indicated lipid peroxidation. Thiobarbituric acid reactive substance (TBARS) is one of methods for analyzed MDA by precipitated protein by TCA. The TBA-MDA reaction which one molecule of MDA reacts with two molecules of TBA $(TBA)_2$-MDA in boiling and acetic acid condition avoid its reaction with other substances. This TBA-MDA reaction product is fluorescent and resulting pink color which can be extracted with butanol then measured fluorometrically using spectrofluorometer with excitation and emission wavelengths at 515 and 553 nm, respectively.

Reagents

1. 10% Trichloroacetic acid (TCA) (Sigma Chemical Co., St. Louis, MO, USA)
2. 8% Sodium dodecyl sulfate (SDS)
3. 5 mM Ethylene diamine tetraacetic acid-Na salt (EDTA)
4. 100 mM Buthylated hydroxytoluene (BHT) (Sigma Chemical Co., St. Louis, MO, USA)
5. Ethanol
6. 1.2 % Thiobarbituric acid (TBA) (Sigma Chemical Co., St. Louis, MO, USA)
7. 1,1,3,3-tetramethoxypropane or tetrathoxypropane (TEP)
8. 1.15% KCl

Reagent preparation

1. 10% TCA

   - Weighted 10 g of 10% TCA in volumetric flask 100 ml then diluted with DI water to the volume.

2. 8% SDS

   - Weighted 8 g of 8% SDS in volumetric flask 100 ml then diluted with DI water to the volume.

3. 5 mM EDTA

   - Weighted 0.1861 g of 5 Mm EDTA in volumetric flask 100 ml then diluted with DI water to the volume.

4. 100 mM BHT

   - Weighted 2.2 g of 100 Mm BHT in volumetric flask 100 ml then diluted with absolute ethanol to the volume. This reagent was kept in 4°C.

5. 1.2 % TBA

   - Weighted 0.15 g of 1.2 % TBA in 25 ml of DI water then warmed in water bath 100 °C for 1 hour.

6. TEP standard or 1,1,3,3-tetramethoxypropane (1 M TEP=1M MDA, Freshly prepare)

   - The standard was diluted to the concentration of 1, 0.8, 0.4, 0.2 and 0.1 nmol/ml. 10 µl of 4 M of stock standard MDA was diluted in 90 µl of 50% ethanol to get the concentration of 40 nmole/ml. Then 40 µmole/ml of standard was diluted to 200 nmol/ml by taking 125 µl of 40 µmole/ml standard and adding to 25 ml of DI water. 500 µl of 200 nmole/ml standard was added to 10 ml of Normal saline to get 10 nmol/ml standard. The 10 nmole/ml of standard was used to prepare the serial dilution to obtain concentrations of 1, 0.8, 0.4, 0.2 and 0.1 nmol/ml.

Procedure of malondialdehyde (MDA) in plasma

1. Pipette 100 µl of plasma to a clean glass tube and added 25 µl of BHT
2. Added 500 µl of 10% TCA and vortex for 1 min
3. Added 250 of 5 mM EDTA and vortex
4. Added 250 µl of 8% SDS and 750 µl of 0.6% TBA then vortex for 1 min
5. Heated 100 °C for 1 hour and cooled in cool water to room temperature
6. Extract with butanol (1:1)
7. Centrifuged 3,500 rpm at 25°C for 10 min

8. The color supernatant was analyzed by spectrofluorometer (Perkin Elmer LS 55 luminescence spectrometer) with 515 nm excitation and 553 nm emission filters.

Procedure of hepatic malondialdehyde

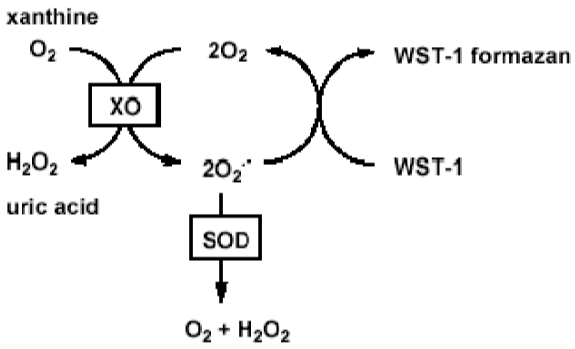
1. Prepared 0.2 g liver
2. Homogenized (1:10 W:V) in 2 ml of 1.15% KCl and 100 µM of 100 mM BHT
3. Pipette 0.5 µl of homogenate and added DI 500 µl
4. Added 1 ml of 10% TCA and vortex for 1 min
5. Added 500 µl of 5 mM EDTA and vortex
6. Added 500 µl of 8% SDS and 1,500 µl of 1.2% TBA then vortex for 1 min
7. Heated 100 °C for 1 hour and cooled in cool water to room temperature
8. Centrifuged 3,500 rpm at 25°C for 10 min
9. The color supernatant was analyzed by spectrofluorometer (Perkin Elmer LS 55 luminescence spectrometer) with 515 nm excitation and 553 nm emission filters.

APPENDIX D

DETERMINATION METHOD OF SUPEROXIDE DISMUTASE IN MICE PLASMA

In this study, superoxide dismutase was measured using superoxide dismutase activity assay kit (Biovision Laboratory, Switzerland). Superoxide dismutase (SOD) is one of the most important enzymatic antioxidant. It catalyzes the dismutation of the superoxide anion ($2O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). The sensitive SOD assay kit utilizes a highly water-soluble tetrazolium salt (WST-1 or (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) that produces a water-soluble formazan dye upon reduction with superoxide anion. In addition, the rate of the reduction with a superoxide anion is linearly related to the xanthine oxidase (XO) activity and is inhibited by SOD as shown in Figure 7.5. Therefore, the inhibition activity of SOD can measured using colorimetric method.

Figure 7.5 Superoxide dismutase and WST-1



APPENDIX E
DETERMINATION METHOD OF TOTAL CoQ10 CONTENT IN MICE PLASMA

Principle

Total CoQ_{10} content derived from the plasma extraction with ethanol/ hexane (ratio 1: 2.5 v/v) to be lipophilic phase of CoQ_{10} from modified Okamoto's method (ref). Lipophilic phase of CoQ_{10} was extracted into hexane layer. The oxidized CoQ_{10} form was transformed to reduced CoQ_{10} form by sodium borohydride. Then, total CoQ_{10} content was determined by HPLC-ECD (high performance liquid chromatography with electrochemical detector).

Reagent

1. Hexane (Nacali Tesque, Kyoto, Japan)
2. Absolute ethanol (Nacali Tesque, Kyoto, Japan)
3. CoQ_{10} Standard stock (Eisai Co., Ltd., Tokyo, Japan)
4. Internal standard : CoQ_7 standard stock (Eisai Co., Ltd., Tokyo, Japan)
5. Sodium borohydride ($NaBH_4$; Nacali Tesque, Kyoto, Japan)
6. Methanol (Nacali Tesque, Kyoto, Japan)
7. Acetonotrile (Nacali Tesque, Kyoto, Japan)
8. 70% Perchloric acid (MERCK, Merck KGaA, Darmstadt, Germany)
9. Sodium Perchlorate Monohydrate (Wako Pure Chemical Industries, Ltd.)

Standard preparation

1. **Stock solution (100 µg/ml and 10 µg/ml) :**

   - 100 µg/ml concentration: 0.010 g of CoQ10 or CoQ7 standard stocks was completely dissolved in 100 ml ethanol.

- 10 µg/ml concentration: diluted 10 ml of 100 µg/ml CoQ10 or CoQ7 in 100 ml ethanol.

2. **Preparation of reducing agent (0.25% Sodium borohydride/$NaBH_4$) :**
   Reducing agent was prepared freshly by weighing 0.0025g of $NaBH_4$ and dissolved by 100 µL deionized water and added 900 µL ethanol, mixed well.
3. **Reduced standard CoQ_{10} and CoQ_7 (1 µg/ml)**
   Pipette 100 µl of 10 µg/ml stock CoQ_{10} or CoQ_7 solution into dark brown tube then added 780 µl absolute ethanol and 20 µl of 0.25% $NaBH_4$ solution, stand for 15 min to complete reduction before HPLC injection.

Procedure

1. Pipette 100 µl of plasma into two dark brown tubes (duplicate analysis).
2. Added 100 µl of an internal standard CoQ_7 (10 µg/ml) into one of two tube, then added 3.5 ml ethanol/hexane (ratio 1:2.5 v/v) into the both tubes.
4. Shake vigorously with shaker machine for 10 min.
5. Centrifuged at 3,000 rpm for 10 minutes at 5°C.
6. Pipette 2 ml hexane phase into another dark brown tubes and evaporated hexane phase under nitrogen and then dried solution was resolvent with absolute ethanol and 0.25% $NaBH_4$ solution, mixed well.
7. CoQ_{10} content determined by HPLC-ECD.

HPLC condition for CoQ_{10} analysis

Column:	Finepak SIL C18-5 stainless-steel column 250 mm x 4.6 mm (JASCO corporation, Japan)
Flow rate:	0.8 ml/ min
Detector:	An electrochemical detector: 807-IT, Jasco Co, Ltd., Japan
Injection volume:	20 µl
Column Temp Control:	37 °c

Preparation of mobile phase for HPLC:

Mobile phase was prepared by dissolving 0.05 M sodium perchlorate in ethanol-methanol-acetonotrile-70% perchloric acid (400:300:300:1, v/v).

Calculation:

Total CoQ_{10} or total CoQ_7 content was calculated by

$$\text{CoQ content (ng/ml)} = \frac{A \times C \times E}{B} \times 1000$$

A= Sample peak area

B= Standard CoQ peak area

C= Dilution volume

D= Sample volume

Quality assurance of the analytical results

The difference of analytical results in between two tubes (duplicate analysis) was accepted at $\leq$ 5% in each sample while, the percent recovery of internal standard (CoQ7) was accepted at $\geq$ 95% in each sample.

APPENDIX F

DETERMINATION METHOD OF VITAMIN E IN MICE PLASMA

Principle

Vitamin E is most effective lipid-soluble vitamin and antioxidant that protect lipid peroxidation. Vitamin E is saponified with hexane to remove lipid and separated out vitamin E then dried hexane, dissolved the residue with mobole phase and determined by HPLC-mobile phase solvent.

Reagent

1. 0.5% Tetrahydrofuran
2. Acetonitrite (CH_3CN)
3. Triethylamine (TEA)
4. Ammonium acetate
5. Methanol
6. Standard vitamin E

Procedure

1. Pipette 150 µl of plasma in DI water 150 ml and mixed
2. Added 150 µl of internal standard (retinyl acetate 150 ml) and mixed
3. Added 750 µl of hexane and mixed for 30 sec.
4. Centrifuged at 3,500 rpm for 3 minutes.
5. Pipette 750 µl hexane phase into another dark brown tubes
6. Re-extract the sample with 750 µl hexane
7. Centrifuged at 3,500 rpm for 3 minutes.
8. Evaporated hexane phase under nitrogen
9. Re-dissolved with 150 µl of mobile phase
10. Determined 60 µl of sample by HPLC

HPLC Condition for vitamin E

Column:	SUPELCOSIL LC-18 (4.6×250 nm, 5 µm)
Flow rate:	1.5 ml/ min
Detector:	UV-Vis at 294 nm
Injection volumn:	60 µl

Preparation of mobile phase for HPLC:

Mobile phase was prepared by ammonium acetate: methanol: Tetrahydrofuran (THF): acetonitrite (CH_3CN): Triethylamine (TEA) (0.02: 6: 14: 80: 0.10, v/v)

APPENDIX G
DETERMINATION METHOD OF FREE CHOLESTEROL AND CHOLESTERYL ESSTERS (CEs) IN MICE PLASMA

Reagent

1. Free Cholesterol (FC)
2. Cholesteryl linolenate (CLN)
3. Cholesteryl Arachidonate (CA)
4. Cholesteryl linoleate (CL)
5. Cholesteryl Oleate (CO)
6. Cholesteryl Palmitate (CP)
7. Acetonitrite
8. Isopropanol (IPN)

Procedure

1. Pipette 100 µl of plasma into a clean glass tube
2. Extracted with 500 µl of methanol, 2.5 ml of hexane and mixed for 1 min
3. Centrifuged at 1,700 rpm at 4 ℃ for 5 min
4. Pipette 2 ml of supernatant (hexane layer) to new test tube
5. Evaporated under nitrogen
6. Re-dissolved with 400 µl of mobile phase
7. Determined 20 µl of sample by HPLC injection

HPLC Condition for free cholesterol and cholesteryl esters (CEs)

Column:	Hypersil BDS C18 (4.6×250 nm, 5 µ)
Mobile phase:	Acetonitrite: Isopropanol (75: 25, v/v)
Flow rate:	1.2 ml/ min
Detector:	Free cholesterol, Cholesteryl esters: a Waters 996 photodiode array UV at 210 nm

Injection volumn: 20 µl

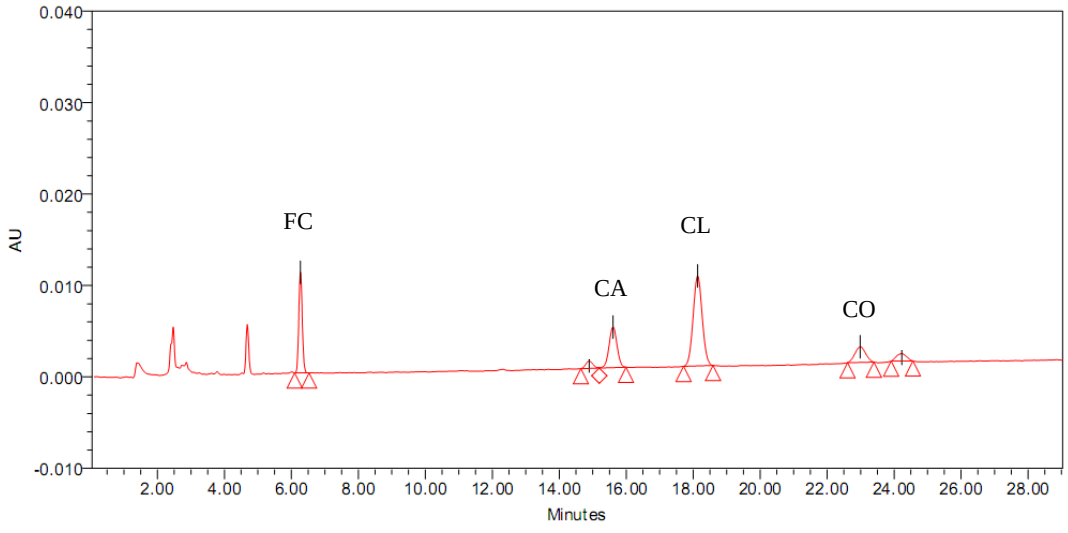
Scale time: 35 min

Column Temp Control: 50°c

Table 7.1 Preparation of stock substance, volumn and final concentration standard for free cholesterol and cholesterol esters analyzing

	Stock (µg/ml)	**Vol (µl)**	**Final concentration (µg/ml)**
FC	1,000	90	180
CLN	1,000	5	10
CA	1,000	15	30
CL	1,000	180	360
CO	1,000	90	180
CP	1,000	60	180
IPN	1,000	65	10

Figure 7.6 Chromatogram of free cholesterol (FC) and cholesteryl esters (cholesteryl arachidonate (CA), Cholesteryl linoleate (CL), Cholesteryl Oleate (CO), Cholesteryl Palmitate (CP)) in standard solution. Retention time of FC= 6, CA= 15, CL= 18, CO= 22 min.



APPENDIX H
DETERMINATION METHOD OF BLOOD SMEAR AND STAINING WITH WRIGHT'S STAIN TO EVALUATE RED BLOOD CELL MORPHOLOGY

Principle

A small drop of blood was placed near the end of a glass slide. A second slide was used as a spreader. The blood was spread in a thin film over the slide as monolayer, allowed to air-dry and stained with Wright's stain. Wright's stain was an alcoholic solution of methylene blue and eosin y that facilitated the differentiation of blood cell types which examined under a light microscope (120).

Reagents

1. Glass slide, 3 × 1 inch
2. Wright's stain
3. Deionized water
4. Paper towel

Procedure

1. Placed a small drop of blood near the end of a glass slide.
2. Placed an edge of second slide as a spreader on the specimen slide in front of the blood drop. Hold the spreader slide at a 30 angle and drawn it back against the drop of the blood. Allowed the blood spread almost to the edge of the slide. Push the spread forward with one light and smooth motion to produce smear and labeled no. of mice
3. Allowed the blood film to air-dry.
4. Fixed by absolute methanol for 1 min.
5. Stain by Wright's stain for 5 min.
6. Wash by deionizer water to rinse of the excess stain.

7. Wide off excess fluid from the back of the slide. Placed the slide upright on a paper towel and allowed to air-dry.
8. Examine the smear with the microscope

APPENDIX I

DETERMINATION METHOD OF MEASUREMENT HEPATIC IRON CONTENT

Hepatic iron content or tissue iron concentration (TIC) measured non-heme iron directly from liver autopsy or liver biopsy which is the marker to indicate the severity of β-thalassemia patient. In briefly, weighted 1 g of lyophilized liver tissue to erlenmeyer flask then added 10 ml of acid solution and covered. Incubated at 65 °C for 20 hours and cooled to room temperature. Pipette 0.2 ml of supernatant (clear yellow solutions) to new test tube, added 10 ml of working chromagen reagent and mixly then kept at room temperature at least 10 min and determined the optical density of solutions by spectrophotometer at wavelength 535 nm. A standard solution was used as 2 µg/ml of iron standard. Hepatic iron content can be calculated in according to the equation:

$$\text{Hepatic iron content} = \frac{\text{OD tissue extract - OD blank}}{\text{OD iron standard – OD blank}} \times \frac{200}{\text{tissue weight (g)}}$$

Table 7.2 Reactive Oxygen Species by flow cytometric analyses of the fluorescence of H_2O_2-stimulated (D+H) and without H_2O_2-stimulated at min 0 and 20 (D-H) of Red blood cells in mice

Group	Code Number	D+H		D-H	
		min		min	
		0	20	0	20
NC	1.10	29.25	61.32	25.49	31.87
NC	1.11	24.19	59.78	20.53	46.48
NC	1.90	29.31	83.31	19.5	31.22
NC	5.45	24.44	52.48	27.89	39.84
NC	5.60	41.81	84.06	27.58	41.7
Mean ± SD	**N = 5**	**29.80 ± 7.16**	**68.19 ± 14.54**	**24.20 ± 3.95**	**38.22 ± 6.56**
NR	1.13	25.98	47.77	24.43	29.49
NR	1.14	26.5	53.11	18.68	20.43
NR	1.15	24.62	72.12	19.8	22.41
NR	1.16	28.39	61.05	21.68	24.8
NR	5.47	22.05	90.64	17.72	26.72
Mean ± SD	**N = 5**	**25.51 ± 2.36**	**64.94 ± 17.04**	**20.46 ± 2.66**	**24.77 ± 3.55**
TC	1.73	56.29	237.64	50.48	48.43
TC	1.76	50.31	166.66	49.32	51.28
TC	2.55	45.18	213.74	52.2	66.24
TC	2.62	71.6	154.68	45.18	213.74
Mean ± SD	**N = 4**	**55.85 ± 11.44**	**193.18 ± 39.09**	**49.30 ± 2.99**	**94.92 ± 79.60**
TL	1.82	37.02	142.34	42.51	63.01
TL	1.84	54.8	176.3	52.52	53.8
TL	1.90	46.89	179.11	57.05	83.33
Mean ± SD	**N = 3**	**46.24 ± 8.91**	**165.92 ± 20.47**	**50.69 ± 7.44**	**66.71 ± 15.11**
TH	1.78	53.94	160.81	48.73	40.62
TH	1.79	58.85	253.76	46.95	51.93
TH	2.56	42.32	189.05	45.88	58.91
Mean ± SD	**N = 3**	**51.70 ± 8.49**	**201.21 ± 47.65**	**47.19 ± 1.44**	**50.49 ± 9.23**

Table 7.3 Parameters for lipid fluidity at the different regions in red blood cell membranes in mice

Group	Code number	Lipid fluidity			
		Order parameter (S)	τ_B ($\times 10^{10}$ s)	τ_C ($\times 10^{10}$ s)	ho/h-1
NC	1.30	0.71	18.82	22.98	4.34
NC	1.40	0.73	21.65	25.90	4.94
NC	1.50	0.70	18.51	22.53	4.36
NC	1.70	0.72	18.38	22.26	4.38
NC	5.22	0.72	18.34	22.83	4.49
NC	5.54	0.71	17.69	22.33	4.33
NC	5.62	0.72	18.70	22.87	4.41
NC	5.93	0.70	17.66	21.29	4.27
NC	5.94	0.71	16.68	21.00	4.18
Mean ± SD	**N = 9**	**0.71 ± 0.01**	**18.49 ± 1.36**	**22.67 ± 1.40**	**4.41 ± 0.22**
NR	1.20	0.71	18.81	22.78	4.51
NR	1.60	0.73	19.79	23.34	4.54
NR	5.23	0.72	18.74	23.28	4.38
NR	5.24	0.70	19.00	23.05	4.56
NR	5.52	0.70	17.46	21.64	4.36
NR	5.53	0.70	17.62	21.93	4.54
NR	5.91	0.73	17.12	21.87	4.48
NR	5.92	0.71	16.14	20.56	4.08
NR	6.10	0.72	17.98	21.98	4.23
Mean ± SD	**N = 9**	**0.71 ± 0.01**	**18.07 ± 1.20**	**22.27 ± 0.92**	**4.41 ± 0.16**

Table 7.3 Parameters for lipid fluidity at the different regions in red blood cell membranes in mice plasma (continued)

Group	Code number	Lipid fluidity			
		Order parameter (S)	**τ_B ($\times 10^{10}$ s)**	**τ_C ($\times 10^{10}$ s)**	**ho/h-1**
TC	1.40	0.73	17.77	22.32	4.72
TC	1.51	0.70	18.48	23.02	4.73
TC	4.50	0.71	17.28	22.47	4.63
TC	4.57	0.71	20.01	25.28	4.69
TC	4.58	0.72	17.32	21.89	4.72
TC	4.61	0.71	17.69	24.10	4.70
Mean ± SD	**N = 6**	**0.71 ± 0.01**	**18.09 ± 1.03**	**23.18 ± 1.28**	**4.70 ± 0.04**
TL	1.10	0.71	16.32	20.57	4.49
TL	1.19	0.73	16.95	21.39	4.22
TL	1.20	0.71	18.60	23.09	4.42
TL	1.46	0.71	19.32	23.72	4.61
TL	4.54	0.72	18.65	23.06	4.53
TL	4.62	0.71	17.19	21.95	4.49
TL	4.70	0.72	19.14	23.85	4.85
Mean ± SD	**N = 7**	**0.72 ± 0.01**	**18.02 ± 1.18**	**22.52 ± 1.24**	**4.52 ± 0.19**
TH	1.35	0.72	17.57	22.37	4.76
TH	1.38	0.71	16.71	21.17	4.40
TH	1.47	0.70	19.52	23.87	4.62
TH	4.71	0.68	17.99	22.07	4.48
TH	4.72	0.70	16.72	20.97	4.42
TH	4.86	0.71	18.28	23.22	4.69
TH	4.91	0.73	18.11	22.78	4.86
Mean ± SD	**N = 7**	**0.71 ± 0.02**	**17.84 ± 0.98**	**22.35 ± 1.05**	**4.61 ± 0.18**

Table 7.4 The level of malondialdehyde (MDA) in mice (µmol/L)

Group	Code number	MDA (µmol/L)
NC	1.10	0.016
NC	1.11	0.010
NC	1.30	0.018
NC	1.40	0.018
NC	1.70	0.019
NC	1.90	0.011
NC	5.45	0.012
NC	5.54	0.015
NC	5.60	0.008
NC	5.62	0.013
NC	5.93	0.017
NC	5.94	0.017
Mean ± SD	**N = 12**	**0.014 ± 0.004**
NR	1.13	0.010
NR	1.14	0.007
NR	1.15	0.012
NR	1.16	0.019
NR	1.20	0.014
NR	5.23	0.017
NR	5.47	0.007
NR	5.52	0.014
NR	5.53	0.013
NR	5.91	0.016
NR	5.92	0.014
Mean ± SD	**N = 11**	**0.013 ± 0.004**
TC	1.40	0.023
TC	1.51	0.016
TC	1.73	0.014
TC	1.76	0.005
TC	2.55	0.006
TC	2.62	0.009
TC	4.57	0.015
TC	4.61	0.019
Mean ± SD	**N = 8**	**0.013 ± 0.006**
TL	1.10	0.019
TL	1.19	0.013
TL	1.46	0.015
TL	1.82	0.006
TL	1.84	0.004
TL	1.90	0.004
TL	4.54	0.012
TL	4.62	0.014
TL	4.70	0.010
Mean ± SD	**N = 9**	**0.011 ± 0.005**

Table 7.4 The level of malondialdehyde (MDA) in mice (µmol/L) (continued)

Group	Code number	MDA (µmol/L)
TH	1.38	0.014
TH	1.47	0.007
TH	1.78	0.006
TH	1.79	0.004
TH	2.56	0.004
TH	4.71	0.009
TH	4.72	0.011
TH	4.86	0.015
Mean ± SD	**N = 8**	**0.009 ± 0.004**

Table 7.5 Superoxide dismutase in plasma of mice (U/ml)

Group	Code number	SOD (U/ml)
NC	1.50	5
NC	1.70	15
NC	5.22	10
NC	5.62	10
NC	5.93	15
NC	5.94	0
Mean ± SD	**N = 6**	**9.17 ± 5.85**
NR	1.20	0
NR	5.24	0
NR	5.53	15
NR	5.91	5
NR	5.92	10
NR	6.10	15
Mean ± SD	**N = 6**	**7.50 ± 6.89**
TC	1.51	20
TC	4.57	10
TC	4.58	20
TC	4.61	15
Mean ± SD	**N = 4**	**16.25 ± 4.79**
TL	1.10	20
TL	1.20	20
TL	1.46	20
TL	4.54	15
Mean ± SD	**N = 4**	**18.75 ± 2.50**
TH	1.35	5
TH	4.71	20
TH	4.72	5
TH	4.86	20
Mean ± SD	**N = 4**	**12.50 ± 8.66**

Table 7.6 Total CoQ_{10} content in mice plasma (ng/ml)

Group	Code number	CoQ_{10} levels (ng/ml)
NC	1.30	31.09
NC	1.40	24.53
NC	1.50	24.99
NC	1.70	27.92
NC	5.22	29.44
NC	5.54	21.16
NC	5.62	19.04
NC	5.93	32.02
NC	5.94	22.97
Mean ± SD	**N = 9**	**25.91 ± 4.50**
NR	1.20	32.17
NR	1.60	32.97
NR	5.23	37.43
NR	5.24	32.80
NR	5.52	35.86
NR	5.53	27.49
NR	5.91	31.38
NR	5.92	23.42
NR	6.10	34.63
Mean ± SD	**N = 9**	**32.02 ± 4.29**
TC	1.40	19.07
TC	1.51	15.33
TC	4.50	17.10
TC	4.57	16.37
TC	4.58	15.14
TC	4.61	22.82
Mean ± SD	**N = 6**	**17.64 ± 2.91**
TL	1.10	18.05
TL	1.19	24.79
TL	1.20	26.29
TL	1.46	16.78
TL	4.54	19.60
TL	4.62	19.62
TL	4.70	23.08
Mean ± SD	**N = 7**	**21.17 ± 3.58**
TH	1.35	25.50
TH	1.38	19.92
TH	1.47	24.07
TH	4.71	19.39
TH	4.72	17.79
TH	4.86	28.26
TH	4.91	19.27
Mean ± SD	**N = 7**	**22.03 ± 3.92**

Table 7.7 Vitamin E levels in mice plasma (µg/dl)

Group	Code number	Vit E (µg/dl)
NC	1.10	11.20
NC	1.11	10.60
NC	1.30	8.60
NC	1.40	13.10
NC	1.50	17.70
NC	1.70	12.70
NC	1.90	14.00
NC	5.22	12.50
NC	5.45	7.80
NC	5.54	11.10
NC	5.60	18.00
NC	5.62	11.50
Mean ± SD	**N =12**	**12.40 ± 3.10**
NR	1.13	9.30
NR	1.14	12.70
NR	1.15	8.10
NR	1.16	13.90
NR	1.20	7.40
NR	1.60	14.00
NR	5.23	9.20
NR	5.24	8.50
NR	5.47	14.50
NR	5.52	7.80
NR	5.53	6.70
NR	6.10	17.20
Mean ± SD	**N =12**	**10.78 ± 3.48**
TC	1.51	9.70
TC	1.73	9.90
TC	1.76	13.00
TC	2.55	9.80
TC	2.62	12.70
TC	4.50	7.90
Mean ± SD	**N = 6**	**10.50 ± 1.97**
TL	1.19	9.20
TL	1.46	8.90
TL	1.82	15.10
TL	1.84	13.50
TL	1.90	8.40
TL	4.54	9.70
Mean ± SD	**N = 6**	**10.80 ± 2.79**
TH	1.38	11.60
TH	1.78	9.60
TH	1.79	9.80
TH	2.56	12.50
TH	4.91	12.60
Mean ± SD	**N = 5**	**11.22 ± 1.44**

Table 7.8 Levels of total cholesterol (TC) and triglyceride (TG) in plasma from mice (mmol/L)

Group	Code number	TC (mmol/L)	TG (mmol/L)
NC	1.10	2.17	0.59
NC	1.11	2.22	0.72
NC	1.90	2.20	0.79
NC	5.45	2.22	0.53
NC	5.60	1.91	0.51
Mean ± SD	**N = 5**	**2.14 ± 0.13**	**0.63 ± 0.12**
NR	1.13	2.51	0.64
NR	1.14	2.22	0.75
NR	1.15	2.09	0.54
NR	1.16	2.09	1.04
NR	5.47	1.99	0.60
Mean ± SD	**N = 5**	**2.18 ± 0.20**	**0.71 ± 0.20**
TC	1.73	1.58	0.61
TC	1.76	1.58	0.65
TC	2.55	1.84	0.78
TC	2.62	1.76	0.89
Mean ± SD	**N = 4**	**1.69 ± 0.13**	**0.73 ± 0.13**
TL	1.82	2.17	0.59
TL	1.84	2.12	0.53
TL	1.90	1.78	0.53
Mean ± SD	**N = 3**	**2.03 ± 0.21**	**0.55 ± 0.03**
TH	1.78	1.81	0.38
TH	1.79	1.58	0.52
TH	2.56	2.07	0.38
Mean ± SD	**N = 3**	**1.82 ± 0.25**	**0.43 ± 0.08**

Table 7.9 Levels of free cholesterol (FC) in plasma from mice (mmol/L)

Groups	Code Number	FC (mmol/L)
NC	1.30	0.11
NC	1.40	0.13
NC	1.50	0.16
NC	1.70	0.18
NC	5.22	0.15
NC	5.54	0.08
NC	5.62	0.07
NC	5.93	0.16
NC	5.94	0.19
Mean ± SD.	**N=9**	**0.14 ± 0.04**
NR	1.20	0.15
NR	1.60	0.23
NR	5.23	0.22
NR	5.24	0.20
NR	5.52	0.08
NR	5.53	0.15
NR	5.91	0.25
NR	5.92	0.20
NR	6.10	0.12
Mean ± SD.	**N=9**	**0.18 ± 0.06**
TC	1.40	0.19
TC	1.51	0.20
TC	4.50	0.18
Mean ± SD.	**N=3**	**0.19 ± 0.01**
TL	1.19	0.14
TL	1.20	0.25
TL	4.70	0.15
Mean ± SD.	**N=3**	**0.18 ± 0.06**
TH	1.47	0.01
TH	4.71	0.00
TH	4.72	0.01
TH	4.86	0.01
Mean ± SD.	**N=4**	**0.01 ± 0.00**

Table 7.10 Levels of cholesteryl arachidonate (CA) in plasma from mice (mmol/L)

Groups	Code number	CA (mmol/L)
NC	1.30	0.37
NC	1.40	0.38
NC	1.50	0.57
NC	1.70	0.62
NC	5.22	0.50
NC	5.54	0.40
NC	5.62	0.33
NC	5.93	0.44
NC	5.94	0.55
Mean ± SD.	**N=9**	**0.46 ± 0.10**
NR	1.20	0.64
NR	1.60	0.56
NR	5.23	0.79
NR	5.24	0.74
NR	5.52	0.48
NR	5.53	0.51
NR	5.91	0.56
NR	5.92	0.69
NR	6.10	0.52
Mean ± SD.	**N=9**	**0.61 ± 0.11**
TC	1.40	0.69
TC	1.51	0.59
TC	4.50	0.64
TC	4.57	0.84
TC	4.58	0.73
TC	4.61	0.92
Mean ± SD.	**N=6**	**0.74 ± 0.12**
TL	1.10	0.81
TL	1.19	0.66
TL	1.20	0.79
TL	1.46	0.69
TL	4.45	0.60
TL	4.62	0.74
TL	4.70	0.76
Mean ± SD.	**N=7**	**0.72 ± 0.08**
TH	1.35	0.85
TH	1.38	0.65
TH	1.47	0.72
TH	4.71	0.48
TH	4.72	0.70
TH	4.86	0.94
TH	4.91	0.92
Mean ± SD.	**N=7**	**0.75 ± 0.16**

Table 7.11 Levels of cholesteryl linoleate (CL) in plasma from mice

Groups	Code number	CL (mmol/L)
NC	1.30	0.13
NC	1.40	0.13
NC	1.50	0.27
NC	1.70	0.29
NC	5.22	0.27
NC	5.54	0.14
NC	5.62	0.13
NC	5.93	0.26
NC	5.94	0.33
Mean ± SD.	**N=9**	**0.22 ± 0.08**
NR	1.20	0.23
NR	1.60	0.27
NR	5.23	0.40
NR	5.24	0.39
NR	5.52	0.13
NR	5.53	0.18
NR	5.91	0.36
NR	5.92	0.31
NR	6.10	0.17
Mean ± SD.	**N=9**	**0.27 ± 0.10**
TC	1.40	0.25
TC	1.51	0.34
TC	4.50	0.25
TC	4.57	0.36
TC	4.58	0.29
TC	4.61	0.44
Mean ± SD.	**N=6**	**0.32 ± 0.07**
TL	1.10	0.41
TL	1.19	0.30
TL	1.20	0.37
TL	1.46	0.23
TL	4.45	0.25
TL	4.62	0.36
TL	4.70	0.25
Mean ± SD.	**N=7**	**0.31 ± 0.07**
TH	1.35	0.39
TH	1.38	0.22
TH	1.47	0.26
TH	4.72	0.25
TH	4.86	0.48
TH	4.91	0.45
Mean ± SD.	**N=6**	**0.34 ± 0.11**

Table7.12 Liver and renal profiles in mice serum

Group	**Code number**	**AST (SGOT)** (U/L)	**ALT (SGPT)** (U/L)	**ALK** (U/L)	**Total Protein** (g/dL)	**Albumin** (g/dL)	**Globulin** (g/dL)	**Total bilirubin** (mg/dL)	**Uric acid** (mg/dL)
NC	1.10	62	19	67	4.70	3.10	1.70	0.10	1.70
NC	1.11	140	33	65	4.90	3.20	1.70	0.10	2.50
NC	1.90	497	48	55	4.50	3.10	1.50	0.10	3.20
NC	5.45	125	30	90	5.10	3.10	2.0	0.20	2.50
NC	5.60	119	27	62	4.60	3.00	1.6	0.10	2.30
Mean ± SD	**N = 5**	**188.60 ± 174.92**	**31.40 ± 10.64**	**67.80 ± 13.22**	**4.76 ± 0.24**	**3.10 ± 0.07**	**1.70 ± 0.19**	**0.12 ± 0.04**	**2.44 ± 0.54**
NR	1.13	278	38	58	4.80	3.00	1.80	0.10	2.50
NR	1.14	158	30	64	4.60	2.90	1.70	0.10	2.10
NR	1.15	303	46	67	4.30	2.70	1.60	0.10	5.20
NR	1.16	131	25	67	4.90	3.10	1.80	0.10	5.00
NR	5.47	302	36	97	4.60	3.00	1.60	0.10	2.80
Mean ± SD	**N = 5**	**234.40 ± 83.22**	**35.00 ± 8.00**	**70.60 ± 15.21**	**4.64 ± 0.23**	**2.94 ± 0.15**	**1.70 ± 0.10**	**0.10 ± 0.00**	**3.52 ± 1.47**
TC	1.73	372	49	101	4.60	3.20	1.40	0.20	1.30
TC	1.76	207	27	92	4.70	3.20	1.50	0.20	1.00
TC	2.55	225	31	72	4.70	3.00	1.70	0.20	1.60
TC	2.62	189	44	75	4.50	3.10	1.40	0.10	1.50
Mean ± SD	**N = 4**	**248.25 ± 83.80**	**37.75 ± 10.44**	**85.00 ± 13.83**	**4.63 ± 0.10**	**3.13 ± 0.10**	**1.50 ± 0.14**	**0.18 ± 0.05**	**1.35 ± 0.26**
TL	1.82	141	24	64	4.70	3.00	1.70	0.10	1.40
TL	1.84	129	26	69	4.90	3.30	1.60	0.20	1.80
TL	1.90	491	70	101	4.50	3.10	1.40	0.20	2.30
Mean ± SD	**N = 3**	**253.67 ± 205.62**	**40.00 ± 26.00**	**78.00 ± 20.07**	**4.70 ± 0.20**	**3.13 ± 0.15**	**1.57 ± 0.15**	**0.17 ± 0.06**	**1.83 ± 0.45**
TH	1.78	123	23	92	4.70	3.20	1.50	0.20	0.7
TH	1.79	115	24	104	4.60	3.10	1.40	0.30	1.00
TH	2.56	197	37	88	4.90	3.10	1.80	0.10	2.40
Mean ± SD	**N = 3**	**145.00 ± 45.21**	**28.00 ± 7.81**	**94.67 ± 8.33**	**4.73 ± 0.15**	**3.13 ± 0.06**	**1.57 ± 0.21**	**0.20 ± 0.10**	**1.37 ± 0.91**

Table 7.13 Hepatic iron content (mg Fe/ g dry weight) and hepatic malondialdehyde of mice (µmol/g dry wt.)

Group	Code number	Liver iron (mg Fe/ g dry weight)	Hepatic malondialdehyde (µmol/g dry wt.)
NC	1.10	0.27	0.56
NC	1.11	0.32	0.86
NC	1.30	0.37	1.14
NC	1.40	0.31	1.21
NC	1.50	0.16	0.98
NC	1.70	0.21	0.91
NC	1.90	0.36	1.01
NC	5.22	0.14	0.84
NC	5.45	0.50	1.35
NC	5.54	0.39	1.38
NC	5.60	0.25	0.99
NC	5.62	0.30	1.28
NC	5.93	0.19	1.03
NC	5.94	0.24	1.07
Mean ± SD	**N =14**	**0.29 ± 0.10**	**1.04 ± 0.22**
NR	1.13	0.19	0.92
NR	1.14	0.41	0.93
NR	1.15	0.23	1.03
NR	1.16	0.26	1.04
NR	1.20	0.48	1.33
NR	1.60	0.22	1.19
NR	5.23	0.25	1.11
NR	5.24	0.21	1.15
NR	5.47	0.51	1.26
NR	5.52	0.43	1.28
NR	5.53	0.44	1.31
NR	5.91	0.28	1.17
NR	5.92	0.22	1.13
NR	6.10	0.43	1.23
Mean ± SD	**N =14**	**0.33 ± 0.12**	**1.15 ± 0.13**
TC	1.40	0.96	1.60
TC	1.51	0.62	1.40
TC	1.73	1.53	1.50
TC	1.76	1.40	1.37
TC	2.55	0.89	1.36
TC	2.62	0.61	1.12
TC	4.50	1.26	1.33
TC	4.57	1.11	1.37
TC	4.58	0.85	1.60
TC	4.61	0.75	1.34
Mean ± SD	**N = 10**	**1 ± 0.32**	**1.40 ± 0.14**
TL	1.10	0.84	1.30
TL	1.19	0.92	1.35
TL	1.20	0.72	1.26
TL	1.46	1.73	1.45
TL	1.82	0.77	1.46
TL	1.84	1.33	1.40
TL	1.90	1.12	1.45
TL	4.54	2.11	1.40
TL	4.62	0.47	1.08
TL	4.70	1.47	1.51
Mean ± SD	**N = 10**	**1.15 ± 0.51**	**1.37 ± 0.13**

Table 7.13 Hepatic iron content (mg Fe/ g dry weight) and hepatic malondialdehyde of mice (µmol/g dry wt.) (continued)

Group	Code number	Liver iron (mg Fe/ g dry weight)	Hepatic malondialdehyde (µmol/g dry wt.)
TH	1.35	0.41	1.23
TH	1.38	1.55	1.49
TH	1.47	0.97	1.39
TH	1.78	1.53	1.36
TH	1.79	1.44	1.50
TH	2.56	0.90	1.21
TH	4.71	1.86	1.54
TH	4.72	0.41	1.41
TH	4.86	0.63	1.35
TH	4.91	1.57	1.32
Mean ± SD	**N = 10**	**1.13 ± 0.53**	**1.38 ± 0.11**

Table 7.14 Weight of organs in mg/g body weight

Group	Code number	Liver (mg/g body weight)	Left kidney	Right kidney	Heart	Lung	Spleen
NC	1.10	53.76	6.53	6.53	4.61	6.14	2.69
NC	1.11	53.01	6.48	6.48	3.81	6.10	3.43
NC	1.30	50.00	6.25	4.46	4.46	6.25	3.57
NC	1.40	51.13	6.67	4.89	4.89	7.56	3.11
NC	1.50	52.01	6.12	5.35	4.59	7.65	2.29
NC	1.70	53.14	5.99	6.74	5.61	6.74	3.74
NC	1.90	48.82	6.46	6.46	4.31	6.10	2.87
NC	5.22	60.23	6.44	6.06	5.30	5.30	2.65
NC	5.45	50.57	5.88	6.27	5.49	6.66	4.31
NC	5.54	49.41	5.70	6.18	5.23	9.50	3.80
NC	5.60	49.76	6.22	6.59	5.85	6.22	2.93
NC	5.62	46.82	6.33	5.48	4.64	7.59	2.53
NC	5.93	68.29	5.38	8.28	4.55	6.21	3.31
NC	5.94	50.21	7.66	7.23	5.11	7.23	3.83
Mean ± SD	**N =14**	**52.65 ± 5.49**	**6.29 ± 0.53**	**6.21 ± 0.96**	**4.89 ± 0.57**	**6.80 ± 1.04**	**3.22 ± 0.59**
NR	1.13	52.59	6.82	6.42	4.42	6.82	2.81
NR	1.14	47.78	7.04	7.04	4.81	6.67	2.96
NR	1.16	51.47	6.64	5.81	4.57	7.06	2.91
NR	1.15	54.39	5.88	6.62	5.51	6.62	2.94
NR	1.20	37.95	5.80	4.91	4.02	6.70	4.02
NR	1.60	50.44	7.02	6.14	4.82	6.58	2.19
NR	5.23	56.02	7.58	4.63	4.63	5.90	2.53
NR	5.24	50.35	6.69	5.90	5.90	6.69	2.75
NR	5.47	52.28	6.70	6.70	5.36	8.04	4.02
NR	5.52	53.19	6.88	6.42	5.04	8.25	2.29
NR	5.53	46.75	6.40	5.91	5.91	7.87	3.44
NR	5.91	51.21	6.31	7.05	5.19	5.19	3.34
NR	5.92	50.61	6.06	6.06	4.63	5.35	3.21
NR	6.10	55.64	7.80	7.28	5.72	7.80	4.68
Mean ± SD	**N =14**	**50.76 ± 4.53**	**6.69 ± 0.58**	**6.21 ± 0.76**	**5.04 ± 0.58**	**6.82 ± 0.94**	**3.15 ± 0.70**

Table 7.14 Weight of organs in mg/g body weight (continued)

Group	Code number	Liver (mg/g body weight)	Left kidney	Right kidney	Heart	Lung	Spleen
TC	1.40	53.44	6.04	6.04	6.04	8.83	18.12
TC	1.51	54.83	6.68	6.68	6.68	7.73	11.95
TC	1.73	47.93	6.60	6.16	6.60	6.60	17.15
TC	1.76	56.64	5.86	5.37	7.32	8.30	18.55
TC	2.55	55.76	7.02	5.78	6.61	5.78	14.46
TC	2.62	58.92	5.93	6.36	5.51	5.51	12.29
TC	4.50	54.99	6.01	6.01	6.47	7.86	18.48
TC	4.57	60.17	6.64	5.39	5.39	7.88	12.86
TC	4.58	56.25	5.90	5.90	5.90	7.08	19.67
TC	4.61	43.99	6.28	6.28	6.98	5.59	12.57
Mean ± SD	**N = 10**	**54.29 ± 4.89**	**6.30 ± 0.41**	**6.00 ± 0.41**	**6.35 ± 0.62**	**7.12 ± 1.19**	**15.61 ± 3.07**
TL	1.10	56.95	6.83	6.45	6.83	6.07	9.49
TL	1.19	55.17	6.13	6.13	6.13	6.90	8.81
TL	1.20	59.10	6.75	6.75	6.33	6.75	9.29
TL	1.46	52.33	6.28	5.76	7.85	7.33	16.22
TL	1.82	51.19	5.97	5.97	5.55	7.68	8.96
TL	1.84	56.30	6.67	5.84	6.26	5.84	12.51
TL	1.90	55.45	6.21	6.21	6.21	7.65	19.12
TL	4.54	53.34	6.77	6.77	5.93	8.04	14.82
TL	4.62	62.31	7.42	6.31	6.31	7.79	9.64
TL	4.70	57.57	5.12	4.26	4.69	7.25	14.07
Mean ± SD	**N = 10**	**55.97 ± 3.29**	**6.42 ± 0.62**	**6.05 ± 0.72**	**6.21 ± 0.81**	**7.13 ± 0.74**	**12.29 ± 3.63**
TH	1.35	56.37	6.95	6.56	4.63	6.95	11.58
TH	1.38	49.58	5.89	5.89	7.85	10.80	19.15
TH	1.47	52.14	4.13	4.13	5.16	9.29	13.42
TH	1.78	56.32	6.37	6.37	5.88	7.84	24.00
TH	1.79	57.20	5.82	6.79	8.24	9.69	24.24
TH	2.56	52.49	6.91	6.45	5.99	7.37	11.05
TH	4.71	49.69	8.43	6.21	5.77	7.54	13.75

Table 7.14 Weight of organs in mg/g body weight (continued)

Group	Code number	Liver (mg/g body weight)	Left kidney	Right kidney	Heart	Lung	Spleen
TH	4.72	54.24	6.36	5.51	7.63	7.20	13.98
TH	4.86	66.11	7.44	7.01	5.69	6.57	10.07
TH	4.91	55.76	5.61	6.36	4.87	5.99	12.72
Mean ± SD	**N = 10**	**54.99 ± 4.72**	**6.39 ± 1.16**	**6.13 ± 0.82**	**6.17 ± 1.28**	**7.92 ± 1.52**	**15.40 ± 5.20**

Table 7.15 Body weight of mice

Group	Code number	Sex	Time								
			1	**3**	**5**	**8**	**10**	**12**	**15**	**18**	**21**
NC	1.10	M	23.00	23.60	23.69	22.91	24.77	24.22	25.5	24.77	26.04
NC	1.11	M	24.90	24.94	25.32	24.62	25.71	24.59	26.39	25.99	26.22
NC	1.30	F	19.20	19.85	21.01	19.89	20.69	21.74	21.21	20.52	22.40
NC	1.40	F	18.70	19.42	19.81	19.33	23.24	19.74	20.44	20.74	22.49
NC	1.50	M	22.50	23.25	23.70	23.46	24.01	24.48	24.66	23.92	26.15
NC	1.70	M	24.00	24.76	24.42	24.68	22.04	25.16	25.52	24.88	26.72
NC	1.90	M	27.20	26.79	26.80	25.98	27.22	26.01	27.37	27.56	27.86
NC	5.22	M	24.20	24.39	24.57	24.63	22.89	25.01	24.25	24.35	26.40
NC	5.45	F	25.00	23.80	23.56	23.88	23.92	22.49	25.24	24.27	25.51
NC	5.54	F	19.90	20.15	19.98	20.11	20.52	22.43	21.04	22.10	21.05
NC	5.60	M	25.60	24.90	25.08	24.92	25.33	24.50	25.63	26.11	27.33
NC	5.62	F	20.60	20.62	21.40	21.95	21.92	20.51	22.12	22.85	23.71
NC	5.93	M	22.30	22.16	23.20	24.00	23.01	24.51	25.25	25.01	24.16
NC	5.94	M	21.80	22.10	22.95	23.85	23.45	23.85	24.47	23.69	23.50
NR	1.13	M	24.70	23.82	24.30	24.27	24.66	23.80	24.12	24.47	24.91
NR	1.14	M	26.10	25.90	25.61	25.67	25.20	26.32	25.57	26.19	27.00
NR	1.15	M	27.10	27.14	27.65	26.73	27.15	26.69	26.57	27.17	27.21
NR	1.16	M	22.80	20.10	22.04	22.45	22.71	23.44	23.88	23.88	24.09
NR	1.20	F	20.70	20.60	20.96	20.30	21.59	20.80	21.32	21.20	22.40
NR	1.60	M	22.40	20.15	20.48	21.66	22.39	21.44	21.66	23.30	22.80
NR	5.23	M	24.60	24.37	23.80	24.68	23.49	23.78	24.13	24.37	23.74
NR	5.24	M	25.30	24.06	23.89	24.20	25.22	25.02	24.73	24.95	25.42
NR	5.47	F	22.02	22.07	21.49	22.29	20.60	21.11	22.55	22.66	22.38
NR	5.52	F	20.40	20.78	18.51	20.37	20.12	20.98	21.15	22.15	21.81
NR	5.53	F	19.00	19.20	19.66	19.37	19.58	19.93	19.86	20.35	20.32
NR	5.91	M	23.90	24.01	24.59	25.19	25.11	25.92	26.98	26.68	26.95
NR	5.92	M	23.50	23.48	24.68	26.25	26.00	26.86	27.08	26.43	28.06
NR	6.10	F	18.20	18.01	17.70	18.00	19.20	18.70	18.85	19.50	19.62

Table 7.15 Body weight of mice (continued)

Group	Code number	Sex	Time								
			1	**3**	**5**	**8**	**10**	**12**	**15**	**18**	**21**
TC	1.40	F	18.20	18.29	18.28	19.03	19.42	16.93	20.59	19.45	21.52
TC	1.51	M	23.90	24.62	25.77	26.03	26.60	27.19	27.28	27.86	28.45
TC	1.73	F	21.10	20.15	20.90	21.67	21.11	21.43	21.94	21.04	22.74
TC	1.76	F	19.90	19.42	19.20	19.74	20.05	21.01	20.01	20.46	20.48
TC	2.55	M	19.70	20.24	20.55	21.13	21.50	21.56	23.56	24.40	24.21
TC	2.62	M	20.90	21.24	21.14	21.54	21.74	22.11	22.55	22.81	23.59
TC	4.61	M	24.40	27.48	27.02	27.37	25.01	28.04	27.94	26.79	28.64
TC	4.57	F	23.80	23.37	24.57	23.56	24.69	25.04	23.78	22.92	24.10
TC	4.58	F	24.20	23.74	24.30	23.64	22.61	24.37	24.25	22.13	25.42
TC	4.50	F	20.50	20.13	20.96	21.02	21.45	19.27	22.28	21.96	21.64
TL	1.10	M	28.20	27.79	27.21	27.54	27.12	27.17	27.95	28.10	26.34
TL	1.19	M	24.60	24.24	23.65	25.57	23.67	26.05	25.45	25.40	26.10
TL	1.20	M	23.60	23.21	23.67	24.02	21.69	23.70	23.91	23.40	23.69
TL	1.46	F	19.00	20.13	18.48	19.76	19.58	18.49	19.25	20.18	19.11
TL	1.82	M	23.80	23.11	22.23	23.05	23.35	23.59	24.09	23.96	23.44
TL	1.84	M	23.60	22.05	22.97	22.52	22.13	22.69	23.25	23.81	23.98
TL	1.90	F	19.88	19.60	19.91	19.64	19.51	20.49	20.15	19.88	20.92
TL	4.54	F	23.70	22.51	22.40	23.97	24.12	22.67	22.36	23.13	23.62
TL	4.62	M	25.30	27.01	27.69	26.98	26.48	26.70	27.11	27.30	26.96
TL	4.70	F	23.00	22.54	22.67	22.67	21.22	23.00	22.38	22.51	23.45
TH	1.35	M	25.20	26.03	24.81	24.95	25.44	25.19	25.83	25.63	25.90
TH	1.38	F	19.30	20.01	18.66	18.96	19.00	20.24	21.78	21.69	20.37
TH	1.47	F	17.00	18.43	18.24	17.47	18.01	17.76	18.47	18.75	19.37
TH	1.78	F	19.60	19.50	19.14	19.59	19.55	20.11	20.26	20.58	20.42
TH	1.79	F	19.60	19.70	20.13	20.33	20.30	20.73	19.58	20.17	20.63
TH	2.56	M	21.30	19.66	20.82	20.75	20.70	20.86	21.08	21.59	21.72
TH	4.71	F	22.60	21.77	22.28	21.53	20.21	21.96	21.45	22.01	22.54
TH	4.72	F	23.20	23.14	22.42	22.78	22.46	23.29	23.43	23.62	23.60
TH	4.86	M	22.00	21.71	22.49	23.20	25.16	22.35	21.97	22.48	22.84
TH	4.91	M	26.30	25.61	25.87	25.90	22.01	26.05	25.82	25.35	26.72

BIOGRAPHY

NAME	Miss Kanin Teeratantikanon
DATE OF BIRTH	Dec 4, 1981
PLACE OF BIRTH	Udonthani, Thailand
INSTITUTIONS ATTENDED	Mahidol University, 2000-2004 Bachelor of Nursing Science Mahidol University, 2006-2010: Master of Science (Nutrition)
HOME ADDRESS	414 Ramkhamhaeng 24, Hua mak, Bangkapi Bangkok 10240
E-MAIL ADDRESS	kanint_@hotmail.com