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**ผลผลิตที่ได้ (Output)**

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) พร้อมแจ้งสถานะของการตีพิมพ์ เช่น submitted, accepted, in press, published

   1.1 Saewong, T., Ounjaijean, S., Mundee, Y., Pattanapanyasat, K., Fucharoen, S., Porter, J. B., **Srichairatanakool, S**. (2010) Effects of Green Tea on Iron Accumulation and Oxidative Stress in Livers of Iron-Challenged Thalassemic Mice. *Med. Chem.* 6(2): 57-64. (Correspondent Author, IF = 1.602)

   1.2 **S. Srichairatanakool**, K. Kulprachakarn, K. Pangit, K. Pattanapanyasat, J.B. Porter, S. Fuchaeron. Green tea extract and epigallocatechin 3-gallate reduced labile iron pool and protected oxidative stress in iron-loaded cultured hepatocytes. (Submitted to the Journal of Hepatology, 2011).

   1.3 S. Ounjaijean, S. Fucharoen, J.B. Porter, T. Westermarck, F. Atroshi, **S. Srichairatanakool**. Green tea attenuates erythrocyte oxidative stress in β-thalassemic mice with iron overload. (Submitted to the Acta Haematologica Journal, 2011)

2. การนำผลงานวิจัยไปใช้ประโยชน์

   2.1 เชิงพาณิชย์ ยังไม่มี

   2.2 เชิงนโยบาย (มีการกำหนดนโยบายอิงงานวิจัย/เกิดมาตรการใหม่/เปลี่ยนแปลงระเบียบข้อบังคับหรือวิธีทำงาน)

   2.3 เชิงสาธารณะ (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง) ยังไม่มี

   2.4 เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่) เป็นการศึกษาวิจัยทางคลินิกของแพทย์ประจำบ้านชั้นปีที่ 2 โรงพยาบาลมหาราชนครเชียงใหม่ ที่ มีความสนใจฤทธิ์ทางชีวภาพของชาเขียวและสารสกัดชาเขียวไปให้อาสาสมัครรับประทานเป็นเวลา 2-3 เดือน เพื่อติดตามว่ามีศักยภาพหรือประสิทธิผลช่วยลดระดับกรดยูริคในเลือดได้จริงหรือไม่ และถ้าสามารถลดได้จริงๆ ก็จะนำไปศึกษาในผู้ป่วยโรคเกาต์เพื่อใช้รักษาให้มีระดับกรดยูริคลดลง เพื่อเปรียบเทียบและใช้แทนยารักษาโรคเกาต์คือ allopurinol ต่อไป

3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)

   3.1 รายงานฉบับสมบูรณ์เรื่อง "ศักยภาพของสารคะเตชินจากชาเขียวที่ช่วยลดปริมาณสารประกอบธาตุเหล็กรีดอกซ์ในเซลล์ตับและเซลล์กล้ามเนื้อหัวใจในหลอดทดลองและสิ่งมีชีวิต" จำนวน 10 ฉบับ

   3.2 กนกวรรณ กุลประชากานต์ มานนท์ บุญบางยาง และสมเดช ศรีชัยรัตนกูล เรื่อง "ความเป็นพิษของชาเขียวในเซลล์ตับและเซลล์กล้ามเนื้อหัวใจเพาะเลี้ยง (Cytotoxicity of Green

Tea in Cultured Hepatocytes and Cardiomyocytes)" Proceeding ในงานประชุมวิชาการวิทยาศาสตร์ เกษตร วิศวกรรมและสิ่งแวดล้อม มหาวิทยาลัยพะเยา ครั้งที่ 2 ในวันที่ 25 สิงหาคม 2553 ณ มหาวิทยาลัยพะเยา จังหวัดพะเยา

3.3 การนำเสนอผลงานแบบโปสเตอร์ เรื่อง "Effect of Green Tea on Iron Accumulation and Oxidative Stress in Livers of Iron Challenged Thalassemic Mice" ในการประชุมนักวิจัยรุ่นใหม่ พบ เมธีวิจัยอาวุโส สกว. ครั้งที่ 9 วันที่ 15-17 ตุลาคม 2552 ณ โรงแรมฮอลิเดย์อินน์ รีสอร์ท รีเจ้นท์ บีช ชะอำ จังหวัดเพชรบุรี

3.4 การนำเสนอผลงานแบบโปสเตอร์ เรื่อง "Green Tea Extract and Epigallocatechin 3-gallate Reduced Labile Iron Pool and Protected Oxidative Stress in Iron-Loaded Cultured Hepatocytes" ในการประชุมนักวิจัยรุ่นใหม่ พบ เมธีวิจัยอาวุโส สกว . ครั้งที่ 10 วันที่ 14-16 ตุลาคม 2553 ณ โรงแรมฮอลิเดย์อินน์ รีสอร์ท รีเจ้นท์ บีช ชะอำ จังหวัดเพชรบุรี

3.5 การนำเสนอผลงานแบบบรรยาย ในงานประชุมวิชาการ 1st International Conference on Metal Chelation in Biology & Medicine (MCIBM) เรื่อง "Green tea extract and epigallocatechin-3-gallate reduced labile iron pool and protected oxidative stress in iron-loaded HepG2 cells" วันที่ 11-14 ธันวาคม 2552 ณ University of Bath, Bath, United Kingdom

3.6 ส่วนหนึ่งในวิทยานิพนธ์เรื่อง "Effect of catechins and chitosan on fibrogenesis of liver in mice challenged with iron loading and fat accumulation" ของ Thidarat Saewong M.Sc. Thesis 2009 Chiang Mai University

3.7 ส่วนหนึ่งในวิทยานิพนธ์เรื่อง "Free-radicals scavenging and iron-chelating properties of crude extract and epigallocatechin 3-gallate of green tea in iron-loaded cultured hepatocytes" ของ Kanokwan Kulprachakarn M.Sc. Thesis 2009 Chiang Mai University

3.8 ส่วนหนึ่งในวิทยานิพนธ์เรื่อง "Study of iron overload and oxidative stress using thalassemia and epilepsy model" ของ Sakaewan Ounjaijean Ph.D. Thesis 2010 Chiang Mai University.

**ปัญหาและอุปสรรคที่พบ**

1. หนูทดลองธาลัสซีเมียชนิด BKO และ DH ไม่มีจำหน่ายในประเทศไทย ส่วนหนึ่งต้องขอความอนุเคราะห์จากโครงการวิจัยธาลัสซีเมีย มหาวิทยาลัยมหิดล และอีกส่วนหนึ่งต้องเพาะพันธุ์มาใช้เอง ทำให้มีปริมาณหนูไม่เพียงพอในระหว่างทำการทดลองวิจัย

2. ในระหว่างทำการเหนี่ยวนำให้เกิดภาวะเหล็กเกินในหนูทดลอง มีหนูบางตัวเกิดพยาธิสภาพรุนแรงจนเสียชีวิต ทำให้มีหนูทดลองในบางกลุ่มเหลืออยู่ในจำนวนน้อยกว่าที่วางแผนไว้
3. เนื่องจากหนูเมาส์มีขนาดเล็กและน้ำหนักตัวน้อย การเจาะเลือดจากเส้นเลือดดำบริเวณหางเพื่อนำมาวิเคราะห์ระดับตัวชี้วัดนั้นได้เลือด (whole blood) ออกมาปริมาณน้อยมากเพียง (80-100 ไมโครลิตร) ทำให้ไม่เพียงพอที่จะนำไปใช้วัดปริมาณตัวชี้วัดทางชีวเคมีและทางโลหิตวิทยาได้ครบทุกชนิด โดยเฉพาะอย่างยิ่งปริมาณ free fatty acid และ hydroxycholesterol ที่ต้องใช้พลาสมาปริมาตรมากเพื่อนำไปสกัดด้วยตัวทำละลายอินทรีย์ อย่างไรก็ตามผู้วิจัยได้ทำการตรวจวัดระดับตัวชี้วัดอื่นที่มีความสำคัญเพิ่มเติม
4. หนูเมาส์มีขนาดเล็ก เมื่อนำมาแยกเซลล์กล้ามเนื้อหัวใจ ทำให้ได้ปริมาณเซลล์น้อยมากและไม่เพียงพอสำหรับทำการทดลองกับสารทดสอบหลาย ๆ ตัวได้ในแต่ละครั้ง

## Appendix (ภาคผนวก)

**Reprint 1** เรื่อง "Effects of Green Tea on Iron Accumulation and Oxidative Stress in Livers of Iron-Challenged Thalassemic Mice. T. Saewong, S. Ounjaijean, Y. Mundee, K. Pattanapanyasat, S. Fucharoen, J.B. Porter and **S. Srichairatanakool*** Med Chem 2010; 6(2): 57-64

*Medicinal Chemistry*, 2010, xx, 000 - 00

# Effects of Green Tea on Iron Accumulation and Oxidative Stress in Livers of Iron-Challenged Thalassemic Mice

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**Abstract:** Liver is affected by secondary iron overload in transfusions dependent β-thalassemia patients. The redox iron can generate reactive oxidants that damage biomolecules, leading to liver fibrosis and cirrhosis. Iron chelators are used to treat thalassemias to achieve negative iron balance and relieve oxidant-induced organ dysfunctions. Green tea (GT) (*Camellia sinensis*) catechins exhibit anti-oxidation, the inhibition of carcinogenesis, the detoxification of CYP2E1-catalyzed HepG2 cells and iron chelation. The purpose of this study was to investigate the effectiveness of GT in iron-challenged thalassemic mice. Heterozygous BKO type-thalassemia (BKO) mice (C57BL/6) experienced induced iron overload by being fed a ferrocene-supplemented diet (Fe diet) for 8 weeks, and by orally being given GT extract (300 mg/kg) and deferiprone (DFP) (50 mg/kg) for a further 8 weeks. Liver iron content (LIC) was analyzed by TPTZ colorimetric and Perl's staining techniques. Concentrations of liver reduced glutathione (GSH), collagen and malondialdehyde (MDA) were also measured. Dosages of the GT extract and DFP lowered LIC in the Fe diet-fed BKO mice effectively. The extract did not change any concentrations of liver glutathione, collagen and MDA in the BKO mice. Histochemical examination showed leukocyte infiltration in the nearby hepatic portal vein and high iron accumulation in the livers of the iron-loaded BKO mice, however GT treatment lowered the elevated iron deposition. In conclusion, green tea inhibits or delays the deposition of hepatic iron in regularly iron-loaded thalassemic mice effectively. This will prevent the iron-induced generation of free radicals via Haber-Weiss and Fenton reactions, and consequently liver damage and fibrosis. Combined chelation with green tea would be investigated in β-thalassemia patients with iron overload.

**Key Words:** green tea, thalassemia, iron overload, collagen, malondialdehyde, liver

## INTRODUCTION

Thalassemia patients with iron-overload suffer from endocrinal gland malfunctions, liver fibrosis and cardiac arrhythmia [1]. Eventually, this condition will be fetal unless suitable iron chelation therapy is managed [2]. When transferrin in thalassemia plasma is fully saturated with iron, a toxic form of iron called nontransferrin-bound iron (NTBI) is detectable [3]. The NTBI is taken up increasingly in cardiac myocytes, HepG2 cells and rat primary hepatocytes [4]. Possible mechanisms of the NTBI uptake could be divalent metal transporter (DMT) [5, 6], L-type calcium channel (LTCC) [7, 8] and Zip14 (a member of SLC39A zinc transporter family [9]. Labile iron pool (LIP) is redox-active and highly increased in iron overload, as a result of changes in iron import and ferritin degradation. It plays a role in free radical generation and is the main target of chelators [10].

In chronic injuries, activated hepatic stellate cells (HSC) display proliferation and fibrogenic myofibroblast phenotype, alpha-smooth muscle actin (α-SMA), enhanced contractibility and a high synthesis of extracellular matrix (ECM) component, especially collagen [11]. Increased collagen synthesis in liver cells leads to fibrosis and eventually cirrhosis. Hepatic parenchymal and Kupffer cells also promote the HSC activation by producing lipid hydroperoxides. Increased hepatic iron concentration (HIC) in β-thalassemia patients is associated with liver toxicity, fibrosis, cirrhosis and carcinogenesis

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[12-15]. Presumably, NTBI could be a tumor promoter in T51B rat liver epithelial cells that can disregulate cell cycle [16]. Additionally, chronic active hepatitis C and an increase of liver iron would be the major causative factors of liver fibrosis [17]. Iron-overload patients with primary hemichromatosis showed raised serum alanine aminotransferase (ALT) activity [18]. Intraperitoneal injection of iron dextran increases amounts of cytosolic hemosiderin and ferritin in the liver of iron-loaded rats. Measurements of liver iron concentration by means of a semi-quantitative inductive device (SQUID), $R2^*$-magnetic resonance imaging (MRI) and Perl's staining are used to evaluate efficacy of chelation therapy [19-21].

Desferrioxamine (DFO) and deferiprone (DFP) chelation can decrease levels of ferritin and NTBI in the plasma compartment and the amount of iron burden in the liver of hereditary sideroblastic anemia patients [22] and β-thalassemia major patients [23]. The main target organs of chelation therapy are the liver and heart which accumulate a large amount of iron in cytosolic ferritin, hemosiderin and transitory pool [24-26]. Green tea (*Camellia sinensis*) contains at least five catechin derivatives including epigallocatechin (EGC), catechin (C), epicatechin (EC), epigallocatechin 3-gallate (EGCG) and epicatechin 3-gallate (ECG), of which EGCG and ECG are the major constituents. Most importantly, anti-oxidative and metal-chelating properties of green tea can protect cells from free-radicals imbalance and toxicity in many pathologic conditions. We investigated if green tea would suppress iron overload and oxidative stress in the livers of thalassemic mice.

## MATERIALS AND METHODS

### Chemicals and Reagents

β-Nicotinamide adenine dinucleotide phosphate (reduced form, NADPH); 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB); 5-sulfosalicylic acid (SSA); thiobarbituric acid (TBA); butylated hydroxytoluene (BHT); 2,4,6-tripyridyl-*s*-triazine (TPTZ) and hydroxylammonium chloride were obtained from Sigma-Aldrich Chemical Company. HPLC-grade acetonitrile, ethylacetate and methanol were purchased from Merck Company. Other chemicals and standards were AnalaR grade or the highest pure chemical availabel. DFP was kindly donated by Dr. Chada Phisalaphong at the Government Pharmaceutical Organization, Bangkok, Thailand.

### Green Tea Preparation

Fresh tea shoots were harvested and immediately dried in a microwave oven (3 minutes, 800 watts, 100°C) [27, 28]. Dry tea leaves were extracted with hot deionized water (80 °C) for 15 minutes and filtered through 0.45-μm membrane (cellulose acetate type, Millipore, Maidstone, England). Green tea (GT) crude extract was dried under a vacuum, and the powder was stored at -20°C till further use. Catechin derivatives in the GT extract were analyzed using a reverse-phase HPLC technique [29]. Conditions of the HPLC include analytical column (Waters SpheroSorb-ODS2, 250x4.7 mm, 5-μm), mobile-phase solvent (0.05% $H_2SO_4$ : acetonitrile : ethyl acetate = 86:12:2, *v/v/v*), a flow rate of 1.0 ml/min and optical detection of eluents at 280 nm. Concentrations of the eluted catechins were determined by comparison with authentic standard catechins. Amounts of the persisting EGC, C, EC, EGCG, ECG and total catechins were 102, 5.1, 53.9, 88.7, 4.3 and 255 mg/g dry weight, respectively.

### Animals and Treatments

Adult male C57/BL6 mice (9-12 months old), wild type ($^{\beta\beta}\beta^{+/+}$)(WT) and heterozygous $\beta^{KO}$ type ($^{\beta\beta}\beta^{\beta-KO}$) (BKO), were provided by Professor Suthat Fucharoen at Thalassemia Research Center, Institute of Science and Technology for Research and Development, Mahidol University, Salaya Campus. The BKO thalassemic mice were generated according to Jamsai and colleagues' technique [30, 31]. The WT mice have normal levels of hemoglobin, SI and ROS production. The BKO mice show decreased hemoglobin level, increased SI and ROS productions, but depressed heart rate variability (HRV) value indicating cardiac complications [32]. Their mild anemia and depressed HRV complications are similar to those observed in human thalassemia intermedia. All animal studies were conducted with approval of the Institutional Animal Care and Use Committee of Faculty of Medicine, Chiang Mai University (Reference Number -Y2548). Mice were housed in stainless steel cages in the conventional clean room where the temperature (25±2 °C) and humidity (60±5%) were maintained on a 12-hours light/dark cycle. The mice had free access to a normal chow diet (control diet) and drinking water *ad libitum*.

WT and BKO mice were fed with a ferrocene-supplemented diet (0.2%, *w/w*) (Fe diet) for 90 days to induce iron overload. Iron status including blood hemoglobin and plasma NTBI levels were detected weekly until the NTBI was detectable. They were randomly divided into three subgroups (4 mice each) for oral administrations of normal saline solution (placebo), GT extract (300 mg/kg/day) and DFP (50 mg/kg body weight) altogether with the Fe diet for another 60 days. At termination, the mice were sacrificed by diethyl ether anesthesia and their livers

were dissected for further quantitative and histochemical analyses.

**Blood Analysis**

Blood samples from the vein of rat tails were collected into anticoagulant tubes (sodium heparin) every month for determination of blood hemoglobin and plasma NTBI concentrations. Hemoglobin concentration was assayed using a cyanmethemoglobin method [33].

Plasma NTBI was measured based on NTA chelation/HPLC technique with slight modification [28]. Briefly, plasma (0.45 ml) was incubated with nitrolotriacetic acid (NTA) solution (80 mM in 5 mM MOPS buffer pH 7.0) (0.05 ml) in a polypropylene tube (Eppendrof 1.5-ml capacity) at room temperature for 30 min. Produced Fe-$(NTA)_2$ complex was separated from plasma proteins by spinning the mixture through a filtration membrane (polysulfone type, 0.5-ml capacity, 30-kD cut-off, NanoSep®, Pall Life Sciences, Ann Arbor, MI, USA) in a temperature-controlled bench-top centrifuge 12,000rpm (10620g) (Hettich Centrifugation Germany), 15 °C for 30 min. The filtrate was analyzed using the HPLC system by the following conditions: PEEK solvent delivery tube; a dual-piston high pressure pump (ConstaMetric® 3500 LDC Milton-Roy Inc., Florida USA); Rheodyne7125 manual injector assembled with 50-µl PEEK loop; a glass column (ChromSep ODS1 type, 100x10 mm, 5 µm; Chrompak International, Middleberg, the Netherlands); mobile-phase solvent (3 mM 1-ethyl-2-propyl-3-hydroxypyrid-4-one in 19% acetonitrile buffered with 5 mM MOPS pH 7.0), a flow rate of 1.0 ml/min, a UV-VIS flow-cell detector (SpecMonitor® 2300, LDC Milton-Roy Inc., Florida USA). Optical density of a red-colored product Fe-$(CP22)_3$ representing NTBI was detected at 450 nm. Data analysis was conducted with BDS software (BarSpec Ltd, Rehovot Israel). The NTBI peak was calculated with a calibration curve made from different iron concentrations (0-16 µM Fe-$(NTA)_2$ prepared in 80 mM NTA pH 7.0 solution.

**Measurement of Liver Iron Concentration**

Non-heme iron in the liver was measured using a colorimetric technique as described by the method established by Fischer and Price [34, 35]. All glassware was cleaned with 6 N. HCl overnight and washed 4-5 times with deionized distilled water; otherwise, plasticware was used. Liver tissues were lyophilized for 24 hours, weighed (expressed as mg dry weight) and digested with a mixture of concentrated sulfuric acid and concentrated nitric acid (1:1, v/v) at 65 °C for 20 hours. Afterwards, the clear yellow solution was transferred to a clean test tube and adjusted to a final volume of exactly 10 ml with deionized water. Tissue iron was reduced with ascorbic acid and reacted with TPTZ at room temperature (25 °C) for 30 minutes to form an intense violet-colored product. OD of the product was measured at 595 nm against reagent solution. Additionally, protein concentration of the liver tissue was estimated according to the Bradford method.

**Analysis of Thiobarbituric Acid Reactive Substance (TBARS) Concentration**

Liver tissue (100 mg) was homogenized in the solution containing 50 mM phosphate buffer pH 2.8 (0.8 ml), methanol (0.1 ml) and BHT (50 ppm) in an ice bath. A 0.5-ml aliquot of the homogenate was transferred to 1.1 ml of 10% (w/v) trichloroacetic acid containing BHT (50 ppm), mixed well and centrifuged at a low speed to achieve clear supernatant. The solution (0.5 ml) was mixed with 0.44 M $H_3PO_4$ (1.5 ml) and TBA solution (0.6%, w/v) (1.0 ml), incubated in a water bath at 90 °C for 30 minutes. The solution was filtered through the 0.45-µm syringe filter and measured for OD of the pink-colored product at 532 nm against reagent blank. A standard curve was constructed by using standard 1,1,3,3-tetramethoxypropane solution. TBARS concentration was determined from the standard curve and reported as the MDA equivalent [36].

**Measurement of Hepatic Glutathione Concentration**

Liver tissue was weighed and deproteinized with 5-sulfosalicylic acid solution (5%). After centrifugation at 3500 rpm, 4 °C for 15 min reduced glutathione (GSH) concentration in supernatant was measured colorimetrically using Ellman's method. Basically, GSH converts 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) to 5-thio-2-nitrobenzoic acid (TNB) while generated oxidized glutathione (GSSG) will be recycled by glutathione reductase and NADPH is converted to GSH. Optical density (OD) of the yellow-colored product TNB was measured at 412 nm against reagent blank.

**Determination of Liver Collagen Concentration**

Dry liver tissue (100 mg) wwas hydrolyzed with 6 N HCl at 60 °C for 24 hr and neutralized with 6 N NaOH. The hydrolysate (100 µl) was incubated with an oxidizing solution (chloramines-T and n-propanol in citrate buffer pH 6.0) (200 µl) at room temperature for 5 min. The mixture was added to Ehrlich's reagent (200 µl) and incubated at 60 °C for 45 min. Optical density of colored product was measured photometrically at 570 nm against reagent blank. Standard hydroxylproline solution (0 - 50µg/ml) was



used to make a calibration curve and determine collagen concentration.

### Histochemical Examination

Another part of the liver tissue was fixed immediately in 10 % neutral buffered formalin, prepared on tissue slides and stained with hematoxylin & eosin (H&E) supravital dye and hydrochloric acid-potassium ferrocyanide (Perl's) solutions. The stained tissues were examined by an expert pathologist and photographed with a digital camera. Pink cytoplasm, red nucleus and Prussian blue iron granules appeared according to Perl's staining [35]. Full cellular detail was obtained from H&E staining.

### Data Analysis

The results were expressed as means±SD. Statistical significance of the data was determined by a Student's *t*-test, $p<0.05$ is considered significant.

## RESULTS

### Blood Analysis

Results in Fig. (1) show that ferrocene-supplemented diet (Fe diet) did not enhance hemoglobin synthesis nor erythropoiesis in WT mice as well as mild anemic BKO mice. Neither GTE nor DFP altered the hemoglobin concentration in the treated mice any more. The Fe diet induced formation of NTBI in plasma of the WT and BKO mice in a time-dependent manner. Expectedly, the GTE and DFP were able to decrease the plasma NTBI concentrations of the treated mice, which the GTE being slightly more effective than the DFP as depicted in Fig. (2).

### Liver Iron Concentration

As shown in Table 1, Fe diet increased the LIC of the WT mice significantly (A = 1.209±0.578 µg Fe/mg protein, 29.13±14.44 ng Fe/mg dry weight).

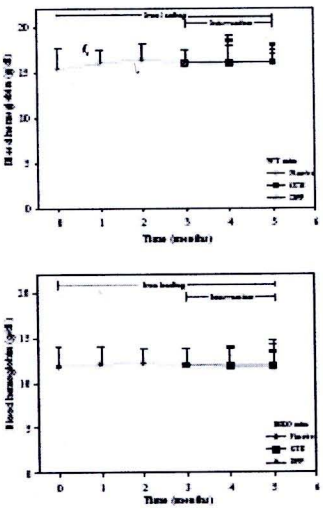


Fig. (1) Blood hemoglobin concentrations of Fe diet-fed WT (top) and BKO (bottom) mice following intervention with placebo, GTE (300 mg/kg) and DFP (50 mg/kg) for 60 days. Data are shown as mean±SD (n = 6).

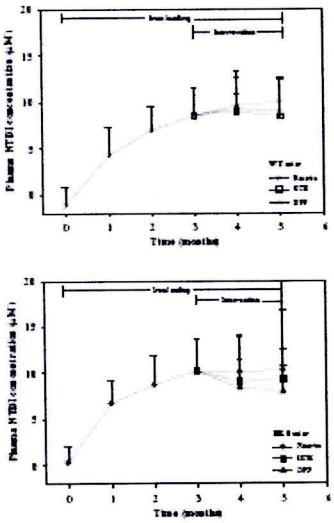


Fig. (2) Plasma NTBI concentrations of Fe diet-fed WT (top) and BKO (bottom) mice following intervention with placebo, GTE (300 mg/kg) and DFP (50 mg/kg) for 60 days. Data are shown as mean±SD (n = 6).

Table 1 Effect of green tea extract (GTE) and deferiprone (DFP) on liver iron concentrations in Fe diet fed-WT and BKO mice. Data are shown as mean±SEM.

| Mice | Diet/ Treatment | Liver iron concentrations | |
|---|---|---|---|
| | | μg Fe/mg protein | ng Fe/ mg dry weight |
| WT | Normal diet | 0.504±0.070 | 9.18±1.15 |
| | Fe diet/Placebo | 1.713±0.648* | 38.31±15.59* |
| | Fe diet/GTE | 0.599±0.042# | 11.03±0.44# |
| | Fe diet/DFP | 0.767±0.111 | 14.51±14.98 |
| BKO | Normal diet | ND | ND |
| | Fe diet/Placebo | 1.950±1.049 | 42.26±24.45 |
| | Fe diet/GTE | 0.335±0.154# | 7.65±3.72# |
| | Fe diet/DFP | 0.595±0.050# | 1.08±0.90# |

*$p$ <0.05 when compared to normal diet; #$p$ <0.05 when compared to Fe diet.

Apparently, GTE lowered the increase of LIC (Δ = -1.114±0.606 μg Fe/mg protein, -27.28±15.15 ng Fe/mg dry weight; $p$ <0.05) and DFP was also effective (Δ = -0.946±0.537 μg Fe/mg protein, -23.80±0.61 ng Fe/mg dry weight). Given an Fe diet, LIC of the BKO mice (1.950±1.049 μg Fe/mg protein, 42.26±24.45 ng Fe/mg dry weight) was slightly higher than that of WT mice (1.713±0.648 μg Fe/mg protein, 38.31±15.59 ng Fe/mg dry weight). GTE and DFP decreased the LIC of Fe diet-fed BKO thalassemic mice (Δ = -1.615±0.895 μg Fe/mg protein and -1.355±0.999 μg Fe/mg protein, respectively) significantly. The result indicates that GTE and DFP exhibited iron-chelating activities and removed iron deposition in the livers of the WT and BKO mice.

Liver TBARS Concentration

The Fe diet markedly increased liver TBAR concentrations (3.08±0.66 μg Fe/mg protein, 0.066±0.015 ng Fe/mg dry weight) when compared to the normal diet (2.34±1.23 μg Fe/mg protein, 0.041±0.020 ng Fe/mg dry weight). Importantly, both GTE and DFP effectively decreased the inclining liver TBARS concentrations in the WT mice (Δ = -0.60 and -0.36 ng Fe/mg dry weight, respectively) as well as the BKO mice (Δ = 0.069 and -0.071 ng Fe/mg dry weight, respectively; $p$<0.05) (Table 2).

Table 2 Effect of GTE and DFP on liver TBARS concentrations in Fe diet fed WT and BKO mice. Data are shown as mean±SEM.

| Mice | Diet/Treatment | Liver TBARS concentrations (MDA equivalent) | |
|---|---|---|---|
| | | μg Fe/mg protein | ng Fe/ mg dry weight |
| WT | Normal diet | 2.34±1.23 | 0.041±0.020 |
| | Fe diet/Placebo | 3.08±0.66 | 0.066±0.015 |
| | Fe diet/GTE | 0.35±0.16# | 0.006±0.003# |
| | Fe diet/DFP | 1.61±0.93 | 0.030±0.017 |
| BKO | Normal diet | ND | ND |
| | Fe diet/Placebo | 3.80±0.09 | 0.080±0.002 |
| | Fe diet/GTE | 0.47±0.29# | 0.011±0.007# |
| | Fe diet/DFP | 0.46±0.07# | 0.009±0.001# |

*$p$ <0.05 when compared to normal diet; #$p$ <0.05 when compared to Fe diet.

The results suggest that GT catechins, particularly EGCG, exhibit iron-chelating properties like DFP that can inhibit the generation of free-radicals. Consequently, the compound can prevent lipid peroxidation and oxidative damage of iron-loaded liver tissue.

Liver Glutathione and Collagen Concentrations

The Fe diet did not influence levels of liver glutathione; nonetheless, GTE and DFP tended to increase the glutathione concentrations of the WT mice (Table 3). Unexpectedly, the GTE and DFP reduced the levels of liver glutathione of the BKO mice. In addition, the Fe diet increased the levels of liver collagen of the WT mice. The GTE and DFP were able to lower the inclining trend of the collagen concentrations in the WT and BKO mice.

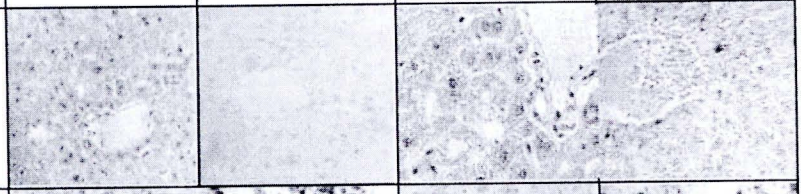
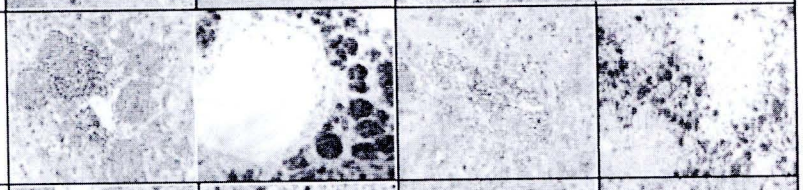
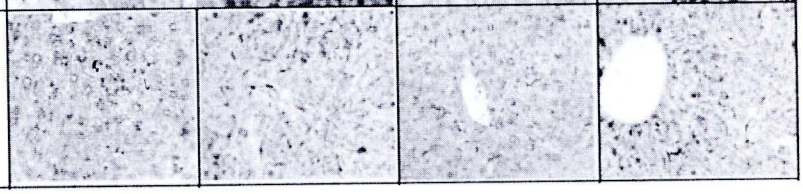
Table 3 Effect of GTE and DFP on liver reduced glutathione (GSH) and collagen concentrations in Fe diet fed WT and BKO mice. Data are shown as mean±SEM.

| Mice | Diet/Treatment | Liver GSH concentration (nmol/mg protein) | Liver collagen concentration (nmol/mg protein) |
|---|---|---|---|
| WT | Normal diet | 3.45±1.25 | 0.023±0.005 |
| | Fe diet/Placebo | 3.95±0.37 | 0.041±0.004 |
| | Fe diet/GTE | 4.04±1.48 | 0.037±0.009 |
| | Fe diet/DFP | 4.28±0.63 | 0.039±0.017 |
| BKO | Normal diet | ND | ND |
| | Fe diet/Placebo | 4.32±0.79 | 0.020±0.002 |
| | Fe diet/GTE | 3.53±0.62 | 0.025±0.003 |
| | Fe diet/DFP | 3.10±0.60 | 0.022±0.008 |

Histochemical Examinations

As shown in Fig. (3) Perl's staining observation showed that Fe diet enhanced iron deposition in liver tissues of both WT and BKO mice. Incredibly, GT was efficient in reducing the iron deposition (less density in Prussian blue granules). As seen in H&E stained tissues, Fe diet enhanced leukocyte infiltration onto liver cells of the WT and BKO mice of the nearby portal vein (H&E staining), indicating potential tissue inflammation. However, the leukocyte infiltration disappeared after the mice were treated with the GTE.

Fig. (3) Hematoxylin & Eosin and Perl's Prussian blue staining results of liver tissues from Fe diet fed-WT and BKO mice treated with GTE.

| Diet/Treatment | WT | | BKO | |
|---|---|---|---|---|
| | H&E stain (x40) | Perl's stain (x40) | H&E stain (x40) | Perl's stain (x40) |
| Normal diet |  | | | |
| Fe diet/Placebo |  | | | |
| Fe diet/GTE |  | | | |

7

## DISCUSSION

As mentioned before, NTBI is present in the plasma of β-thalassemia patients. Our current study has demonstrated that NTBI is detectable in plasma compartments of the Fe diet-fed mice according to the time [37]. However, low concentrations of NTBI can be detected in plasma of the ferric citrate-treated rats [38]. A previous study reported the amount of iron deposition was low in the bone marrow, but high in the liver and spleen of β-thalassemia intermedia. Administration of iron dextran increased blood hemoglobin levels in β-thalssemic mice [39]. However, our two studies observe that ferrocene diet did not increase blood hemoglobin levels in the β-thalassemic mice [37]. Highly accumulated iron in transfusional patients with β-thalassemia can lead to liver inflammation, fibrosis, cirrhosis and functional insufficiency [40]. Splenectomized and cirrhotic thalassemias had higher degrees of iron accumulation and fibrosis than non-splenectomized and non-cirrhotic ones [41]. Hepatocyte siderosis correlates slightly better with chemical liver iron content than does the degree of endothelial and Kupffer cell siderosis [42]. Strong evidence has shown that green tea catechins can inhibit galactosamine- and reactive oxidant-induced hepatotoxicity [43, 44]. Contradictory, intraperitoneal administration of green tea can induce hepatotoxicity in mice [45]. Grege and Lyle found that rats fed a green tea-supplemented normal diet did not experience an alteration alter in liver radio-iron ($^{59}$Fe) concentrations or in gastrointestinal iron absorption [46]. Previous studies have demonstrated that green tea catechins like EGCG, exhibit antifibrogenic activity that can suppress and inhibit collagen synthesis in hepatic stellate cells [47-49]. Omara and colleagues have demonstrated that iron overload impairs macrophage phagocytosis and enhances inflammatory evidence in iron-loaded rats [50]. GTE can decrease focal necrosis and leukocyte infiltration in ischemic/reperfusive livers [51].

In our study, the quantitative and histochemical examinations confirm that green tea extract really lowers the iron accumulation in the livers of WT and BKO mice. Possibly, the metal-binding activity of polyphenolic catechins; especially EGCG and ECG, interfere with luminal iron absorption and also remove intrahepatic iron pools. Green tea catechins counteract iron catalysis in ROS generation via Haber-Weiss and Fenton reactions and lower peroxidative damage of membrane phospholipids. Therefore, levels of liver lipid peroxidation products such as MDA of the GTE-treated mice were less than those of untreated mice. Treatment by antioxidant green tea catechins did not increase levels of intracellular anti-oxidative glutathione as expected. The result implies that catechins only scavenge produced free radicals, but neither enhances the endogenous glutathione synthesis nor recycles oxidized glutathione to reduced glutathione. Increased amounts of collagen reflect to fibrogenesis. The 2-month iron loading may not be sufficient to increase the collagen synthesis in the livers of the WT and BKO mice. Treatment with GT itself would not influence the collagen synthesis either.

Based on these findings, green tea can effectively lower the liver iron concentrations of iron-loaded thalassemic mice by 1) the interference of intestinal iron absorption, 2) chelation of plasma NTBI before intering into liver cells, 3) the mobilization of transient and storage pools of hepatic iron. It can be speculated that green tea extract containing high EGCG and ECG contents could be utilized as phytochemical iron-chelating agents or adjunctive agents with oral iron chelators (eg. deferiprone and deferasirox) in iron-overload patients with β-thalassemia in the future. We are certain that green tea chelation will give compliance, be cost effective and produce minimal side effects to the chelated patients.

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**Manuscript 1**

# Green Tea Attenuates Erythrocyte Oxidative Stress in β-Thalassemic Mice with Iron Overload

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ORIGINAL ARTICLE

**Green Tea Attenuates Erythrocyte Oxidative Stress in β-Thalassemic Mice with Iron Overload**

**S. Ounjaijean[1,5], S. Fucharoen[2], K. Pattanapanyasat[3], T. Westermarck[4], F. Atroshi[5], S. Srichairatanakool[1*]**

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**Abstract**: β-Thalassemia patients suffer from iron overload caused by regular blood transfusions and increased iron absorption. Iron plays a critical role in formation of reactive oxygen species (ROS). Accumulation of nonheme iron on thalassemic erythrocyte membrane triggers an oxidative cascade that leads to their suicidal erythrocyte death. Iron chelators are used for treatment of iron overload and oxidative stress in β-thalassemia patients. Green tea catechins having iron-chelating and antioxidant properties are applied to treat oxidative stress in vitro and in vivo. In this study, we prepared the microwave-processed green tea and investigated if green tea extract (GTE) could ameliorate oxidative stress and prolong survival of red blood cells in iron-loaded thalassemic mice. Mice (C57BL/6J) as wild type (WT) and β-knockout (BKO) thalassemia were fed with ferrocene-supplemented diet (FE diet) for 12 weeks and orally administered with the GTE (300 mg/kg) or DFP (50 mg/kg) for additional 12 weeks. Plasma non-transferrin bound iron (NTBI) was quantified by using HPLC method. Red blood cells labeled with phycoerythrin were detected with flow cytometry. Erythrocyte thiobarbituric acid-reactive substances (TBARS) and hemolysis were measured colorimetrically. Result. Elevated plasma levels of NTBI and lipid peroxidation tended to normalize in response to oral therapy with GTE. At the same time,plasma GSH were increased up to 2-fold. Mice exhibit decreased of lipid peroxidation and improvement in the oxidant–antioxidant balance in erythrocytes. GTE effectively inhibit hemolysis and thereby prolonged RBC half-life. **Conclusion.** Our study demonstrates the ability of GTE to improve several of the fundamental pathological disturbances of Thalassemia. GTE may have clinical potential in diminishing iron overload complication when administered early in disease development. Combined

chelation therapy using GTE with standard iron chelator would be interested in β-thalassemia patients with iron overload.

**Keywords**: green tea, thalassemia, iron overload, oxidative stress, hemolysis, red cell survival

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## 1. INTRODUCTION

Beta-thalassemias are a heterogeneous group of red blood cell (RBC) disorders resulting from reduced or absent synthesis of β-globin chains of haemoglobin. The presence of excess unpaired α-globin chains in RBCs gives rise to aggregates or Heinz bodies, and morphologic changes, causing damage to the RBC membrane. The affected RBCs are prematurely hemolysed in the bone marrow and spleen, resulting in increased RBC turnover, ineffective erythropoiesis, and severe anemia, which can be corrected only by regular blood transfusions [1].

Iron overload, results from enhances iron absorption due to ineffective erythropoiesis and further aggravated by transfusion therapy, is one of the major causes of morbidity and mortality in thalassemic patients. In situations of iron excess the iron-binding capacity of transferrin is highly saturated, resulting in the appearance of nontransferrin-bound iron (NTBI) in the plasma [2, 3]. The toxicity of NTBI is significantly greater than transferrin-bound iron as exemplified by its ability to catalyze free-radical reactions that have life-threatening consequences [4]. NTBI, is thought to catalyze the formation of highly reactive oxygen radicals that generate lipid peroxidation processes, resulting to a breakdown of biomembrane functions and finally leading to cell death.

Administration of iron chelators, desferrioxamine (DFO) or deferiprone (DFP), is commonly promising to prevent or decrease iron-loading burden and accumulation of NTBI in the plasma of β-thalassemia major patients. However, these drugs are issued by being cause of serious side effects, treatment complications and high cost.

Green tea (*Camellia sinensis*) as an excellent source of polyphenols namely catechins, has been shown the protective effect in various oxidative-related pathologic conditions. Our previous studies demonstrated that, green tea catechins contain both anti-oxidative and iron-chelating properties [5, 6], which responsible for the inhibitory effect of green tea extract (GTE) to decreased iron overload and oxidative stress in iron-challenged murine models

[7, 8]. In this study, we investigated if GTE have a protective effect against iron-mediated oxidative damage to the RBCs and prolong RBCs survival of iron-challenged thalassemic mice.

## 2. MATERIALS AND METHODS

### 2.1 Chemicals and Reagents

All HPLC-grade solvents were purchased from Merck Company. Unless other indicated, chemicals and standards were purchased from Sigma-Aldrich Chemical Company with AnalaR grade or the highest pure chemical available. DFP was kindly donated by Dr. Chada Philsalaphong at the Government Pharmaceutical Organization, Bangkok, Thailand.

### 2.2 Green Tea Preparation

Fresh tea shoots were harvested and immediately dried in a microwave oven (3 minutes, 800 watts, 100˚C) [5, 9]. Dry tea leaves were extracted with hot deionized water (80 ˚C) for 15 minutes and filtered through 0.45-μm membrane (cellulose acetate type, Millipore, Maidstone, England). Green tea extract (GTE) was dried under a vacuum, and the powder was stored at -20˚C till further use. Catechin derivatives contained in the GTE were quantitated using a reverse-phase HPLC technique [10]. Conditions of the HPLC include analytical column (Waters SpheroSorb-ODS2, 250x4.7 mm, 5-μm), mobile-phase solvent (0.05% $H_2SO_4$ : acetonitrile : ethyl acetate = 86:12:2, v/v/v), a flow rate of 1.0 ml/min and optical detection of eluents at 280 nm. Concentrations of each catechins were determined by comparison with authentic standard catechins. Amounts of the persisting C, EC, EGC, ECG, EGCG and total catechins in GTE were 68.3, 8.1, 26.9, 9.5, 83.8 and 198.3 mg/g dry weight of tea leaves, respectively.

### 2.3 Animals and Treatments

Adult C57BL/6J mice (initial age 75-120 days), wild type ($^{+/+}\beta^{+/+}$)(WT) and heterozygous β-knock out type ($^{+/+}\beta^{th-3/+}$) (BKO), were provided by Professor Suthat Fucharoen at Thalassemia Research Center, Institute of Science and Technology for Research and Development, Mahidol University. The BKO thalassemic mice were generated by gene targeting technique according to Jamsai and colleagues [11]. Hematological data of BKO mice resemble human Thalassemia intermedia patients with the transfusion-dependent phenotype [12, 13]. All animal studies were conducted with approval of the Institutional Animal Care and Use Committee of Faculty of Medicine, Chiang Mai

University (Reference Number -3/2548). Mice were housed in non-metallic cages in the conventional clean room where the temperature (25±2 °C) and humidity (50±10%) were maintained on 12-hours day/night cycle. The mice had free access to diet and drinking water *ad libitum*.

WT and BKO mice were fed with a ferrocene-supplemented chow diet (FE diet: CP082 diet enriched with 0.2% w/w ferrocene; containing 780 mg Fe/ kg diet) 5 days per week for 12 weeks to induce iron overload. After iron loading period, mice were randomly divided into four subgroups of 12 mice each for oral administrations of deionized water (placebo), GTE (300 mg GT/kg BW/day) and DFP (50 mg/kg BW/day) altogether with the FE diet for another 12 weeks. WT and BKO mice fed with standard diet (CP082; containing 180 mg Fe/kg diet), and administerd with deionized water were served as non-iron loading control.

### 2.4 Blood Analysis

Heparinized blood was collected monthly by tail vein or heart puncture and centrifuged at 3000 rpm, 4 °C for 10 minutes. Erythrocytes were washed and resuspended in PBS, pH 7.4. Plasma was removed and kept frozen at -80 °C for further analysis. Blood hemoglobin concentration was assayed using a cyanmethemoglobin method [14].

Level of total glutathione in erythrocyte and plasma were determined colorimetrically by DTNB method [15] using Glutathione Assay Kit CS0260 (Sigma, St.Louis, MO, USA).

**Determination of reactive oxygen species (ROS).**

Level of ROS generated in red blood cells (RBCs) were determined by flow cytometric assay modified from Amer *et al* [16]. Briefly, 0.4 mM dichlorofluorescein diacetate (DCFH-DA) (Sigma, St. Louis, MO, USA) was added to RBCs suspension ($1\times10^6$ cell/mL in PBS) and incubated at 37°C for 15 min in a humidified atmosphere of 5% $CO_2$. Fluorescence intensity (FI) of DCF from the intact RBCs was analyzed with a Becton Dickinson FACScan (BD Bioscience, CA), and data were analyzed using Cell Quest software (Becton Dickinson). The absolute FI of sample obtained by subtract FI of sample from FI of negative (unlabeled) control for each sample, representing the extent of ROS level in the RBCs. The positive control of each sample had been assessed together by $H_2O_2$ activation.

**Analysis of plasma non-transferrin bound iron (NTBI)**

Plasma NTBI was measured based on NTA chelation/HPLC technique with slight modification [5]. Briefly, plasma (27 µl) was incubated with nitrilotriacetic acid (NTA) solution (final concentration 80 mM in 5 mM MOPS

buffer pH 7.0) at room temperature for 30 min. Produced Fe-(NTA)$_2$ complex was separated from plasma proteins by spinning the mixture through a filtration membrane (polysulfone type, 0.5-ml capacity, 30-kD cut-off, NanoSep®, Pall Life Sciences, Ann Arbor, MI, USA) at 12,000 rpm at 15 °C for 30 min. The filtrate was analyzed using the HPLC system via a glass column (ChromSep ODS1, 100x10 mm, 5 µm; Chrompak International, Middleberg, Netherlands); mobile-phase solvent (3 mM 1-ethyl-2-propyl-3-hydroxypyrid-4-one (CP22) in 19% acetonitrile buffered with 5 mM MOPS pH 7.0), a flow rate of 1.0 ml/min. Optical density of a red-colored product Fe-(CP22)$_2$ representing NTBI was monitored on-line at 450 nm. Data analysis was conducted with BDS software (BarSpec Ltd., Rehovot Israel). The NTBI peak was calculated with a calibration curve made from varied concentrations of 0-16 µM Fe-(NTA)$_2$ prepared in 80 mM NTA, pH 7.0.

**Measurement of lipid peroxidation**

Malondialdehyde (MDA) as index of lipid peroxidation was determined from plasma sample using HPLC-based TBARS assay described by Halliwell and Chirico [17]. Twenty-seven µl of plasma sample was mixed with the reaction mixture, 0.2% BHT solution (3 µl), 0.44 M $H_3PO_4$ (180 µl) and Thiobarbituric acid (0.6% w/v) (60 µl) and incubated at 90°C for 30 minutes. The pink-colored product was filtered through 0.45-µm syringe filter and analyzed with the HPLC system via analytical column (Water Spherosorb ODS2, 250x4.3 mm, 5 µm), mobile-phase solvent of 50 mM $KH_2PO4$: methanol (65:35, v/v) at a flow rate of 1.0 ml/min. Eluents were detected on-line at 532 nm. Plasma TBARS concentrations were determined from the standard curve constructed by varied concentration of 1,1,3,3-tetramethoxypropane and reported as µmol/l of MDA equivalents.

To assess the level of lipid peroxidation in erythrocytes, packed cells were separated from plasma by centrifugation at 3000 x g for 10 min and resuspended in PBS, pH 7.4. To 50 µl of erythrocyte suspension, reaction mixture was added and incubated at 90°C for 30 minutes. Following incubation, it was allowed to stand on ice for 10 min before adding 150 µl tricholoacetic acid (20% w/v). After centrifugation at 12,000 x g for 10 min, the peroxide content in the supernatant was determined by spectrophotometrically at 532 nm. MDA values were evaluated by compared to the absorbance of standard tetramethoxypropane and expressed as pmol/g Hb

**2.5 Red blood cells survival study**

To investigate the effect of green tea catechins on prolong red cell survival, three male mice were randomly taken from each experimental group after iron loading and treatment period. Iron administration and treatment

intervention were continued for 2 month during survival study. *In vivo* biotinylation of the entire RBCs was achieved by tail vein injection of 200 µl, 15 mg/ml EZ-Link Sulfo-NHS-Biotin (Pierce, Rockford, IL, USA). A 2-3 µl blood sample was collected at different period of time by tail vein puncture. Red cells were washed in 10 mmol/l HEPES, pH 7.4, 165 mmol/l NaCl (HBSM), and the number of biotinylated RBCs was determined by incubation of the red cells suspension ($5\times10^6$ cells/ml) with 5 µg/mL phycoerythrin-conjugated streptavidin (BD Bioscience,CA) in HBSM buffer containing 2.5 mmol/l $CaCl_2$. Flow cytometric determination was carried out with FACScan (BD Bioscience,CA) and data were analyzed using Cell Quest software (Becton Dickinson) as described earlier [18, 19]. The RBC survival curve was created for determination of the duration that biotinylated RBCs reduced to 50% ($T_{1/2}$-survival half-time).

### 2.6 *In vitro* assay inhibition of red blood cells hemolysis

The inhibiton of red blood cells hemolysis by GTE or DFP was evaluated by hemolysis test, performed in present of iron chelate and $H_2O_2$ as free radical initiators. RBCs were separated from plasma and buffy coat by centrifugation (1,500 x g, 10 min at 4 °C) and were washed three times by centrifugation (1,500 x g, 5 min) in 10 volumes of 10 mM PBS, pH 7.4. RBCs suspension at 5% hematocrit was preincubated with 50 µl of PBS (negative control), GTE or DFP at different concentrations (1.25 – 20 µg/ml in PBS pH 7.4) for 30 min. Then 100 µl of 100 µM ferric-NTA (in 5 mM MOPS, pH 7.4) and 100 µl of 100 µM $H_2O_2$ (in PBS, pH 7.4) was added. The reaction mixture was shaken gently while being incubated at 37 °C for 3 hr. After incubation, the reaction mixture was diluted with 8 ml PBS and centrifuge at 2,000 x g for 10 min. The resulting supernatant was measured spectrophotometrically at 540 nm. The percentage of hemolysis was calculated by taking hemolysis of hypotonic buffer (PBS, pH 7.4) as 100%. The inhibitory effect of GTE or DFP was compared with standard antioxidant; Trolox (water-soluble tocopherol derivative) (1.25 – 20 µg/ml in PBS pH 7.4).

### 2.7 Data Analysis

Statistical analysis was performed using the SPSS software (Version 16.0, SPSS Inc., Chicago, IL, USA). Data are expressed as mean±SD. The normality of distribution was tested by Skewness/Kurtosis test. Statistical comparison between different groups was performed by using the Student's T Test or Mann-Whitny's U test

depending of the distribution. Bivariate correlation was computed using Pearson's rank correlation. Two-tail significant value was used and a *P* value less than 0.05 was considered as statistically significant.

## 3. RESULTS

### 3.1 Effect of GTE on iron overload and oxidative stress parameters in plasma compartment.

**Plasma NTBI concentration**

The FE diet induced formation of NTBI in plasma of the WT and BKO mice in a time-dependent manner, which BKO mice were more susceptible to iron overload than WT mice. As expected, the increase of NTBI concentration was deteriorated by treatment with GTE and DFP. In contrast with equally effective effect observed in WT mice, GTE was significantly more effective to reduced NTBI concentration than DFP in BKO mice after two months of intervention (**Figure 1**).

**Plasma Lipid peroxidation**

Coincidently with the increase of NTBI in the plasma, mice fed with FE diet have had markedly increase plasma lipid peroxidation in time-dependent fashion (**Figure 2**). GTE showed more effectively to neutralize the plasma MDA concentrations than DFP either in the WT mice or the BKO mice.

**Plasma glutathione content**

Plasma glutathione concentration of FE diet-fed mice was slightly decreased but not statistically significant compared to control mice fed with normal diet (**Figure 3**). Three months administration with GTE, as well as DFP, enhanced the plasma level of glutathione approximately 2-fold and 2.5-fold compared to WT and BKO mice received FE diet with placebo, respectively.

### 3.2 Effect of GTE on hemoglobin level and oxidant-antioxidant status in RBCs compartment.

**Blood hemoglobin concentration**

As shown in (**Figure 4**), the supplementation of iron-enrich diets insignificantly increase hemoglobin level in WT mice as well as mild anemic BKO mice. Neither GTE nor DFP show significant improved of hemoglobin synthesis in the treated mice.

**Erythrocyte ROS formation**

Generation of ROS in erythrocyte of either WT or BKO mice were induced in time-dependent manner by iron administration (**Figure 5**). When administrated with GTE, the iron-induced ROS formation in erythrocytes was neutralized into normal level. In addition, DFP show lower efficacy than GTE to replete erythrocyte ROS in WT mice and had no significant effect in BKO mice.

**Lipid peroxidation of RBCs membrane**

As a consequence of ROS formation, induction of lipid peroxidation in the erythrocyte reflected by the generation of MDA (**Figure 6**). The amount of MDA in the erythrocytes in WT and BKO control group measured were 35.13±4.09 and 48.70±3.65 pmol/g Hb, respectively. Induction of iron overload by FE diet administration to mice results in significantly increased of MDA concentration in the erythrocyte (50.05±6.70 pmol/g Hb in WT mice and 57.30±3.81 pmol/g Hb in BKO mice). The iron-mediated induction of erythrocyte MDA production was efficiently inhibited by treatment with GTE.

**Intracellular glutathione content**

Long term feeding with FE diet cause significantly decreased in levels of erythrocyte glutathione both in WT and BKO mice (**Figure 7**). Treatment with the GTE and DFP could restore the levels of glutathione into baseline level in both types of mice. In addition, the concentration of glutathione in erythrocyte was decreased coincidently with the increased level of erythrocyte MDA ($r = -0.916$, $P < 0.001$).

### 3.3 Red cells survival study

Survival curves plotted in **Figure 8** show that RBC removal was linear for the WT control mice, as reported earlier [18-20]. The number of biotinylated cells decreased approximately 1.9 % per day, with 50% surviving ($T_{1/2}$) at 30 days and an extinction time of 60 days.

In contrast, (**Figure 8, BKO**) thalassemic RBCs demonstrated exponential removal, with both age-dependent and -independent processes. The survival curve was fitted to the equation $A(t) = A_0[1 - (t/T)]e^{kt}$ [21] where A(t) stand for the number of biotinylated RBC at time (t); $A_0$ is the initial number of biotinylated RBC at t = 0; T is the time of senescent death of RBC (extinction time); and k is the sum of the rates of elution of the label and random removal.

Analysis of the exponential curve fit, indicated a more rapid removal of subpopulation of RBCs ($k$ = 0.053) in thalassemic mice as compared to the WT controls with survival half-time ($T_{1/2}$) approximately 17.5 days. In contrast to normal RBCs, by day 30 only 23% of the RBCs of the thalassemic mice were survived. These data indicated that some thalassemic RBCs are removed prematurely, whereas others exhibit normal survival.

Administration with FE diet resulted in the significantly decreased of survival half-time (**Figure 9**). The $T_{1/2}$ in WT mice on FE diet and without iron-chelation treatment are decreased to 23 days, while in BKO mice are decreased to only 13 day.

Treatment with GTE potentially protects red cells from being destroyed by iron-induced oxidative damage. Chelators treated animals had RBC $T_{1/2}$ values intermediate between untreated (placebo group) and non iron-induced controls. The survival rate of WT RBCs in GTE-treated mice were increased approximately 15 % compared to whom received placebo. Furthermore, protective effect of GTE was more predominantly in BKO RBCs, which the survival rate was increased up to 27 % compared to placebo group. As expected, the effect of GTE was slightly more potent than DFP to protect RBCs from senescence.

### 3.4 GTE inhibit hemolysis of RBCs *in vitro*

In order to determine whether GTE can protect red cells from hemolysis caused by iron-catalyzed free radical generation, *in vitro* investigation of hemolysis of RBCs in the present of iron chelate (ferric-NTA) and $H_2O_2$ were performed.

The results revealed that pre-incubation with GTE protect red cells from hemolysis in concentration-dependent manner. At concentration as low as 1.25 µg/ml, GTE can inhibit hemolysis (by 50% in WT RBCs and by 55% in BKO RBCs) compared to the negative control (pre-incubated with PBS). The protective effect of GTE was more potent when compared to iron chelator,DFP or antioxidant;Trolox, taking together.

## 4. DISCUSSION AND CONCLUSION

Thalassemia is a hereditary anemia resulting from genetic defects in hemoglobin production which affects multiple organs and is associated with considerable morbidity and mortality. Lifelong blood transfusion is currently only mainstay treatment to suppress the anemic state, eventually, results in iron overload and consequently oxidative damage of cells and tissues.

Administration of iron dextran had been shown to increase blood hemoglobin levels in β-thalassemic mice [22]. However, we cannot conclude from our result that hemoglobin levels in β-thalassemic mice were increased by ferrocene diet administration. Neither GTE nor DFP cause significant changed in blood hemoglobin level. This result suggested erythropoiesis, either in wild type (WT) or thalassemic (BKO) mice, was not improved in our experimental model.

Iron administration, on the other way, induce iron overload in our experimental mice as determined by the significantly increased of NTBI in the plasma. The toxicity of NTBI to catalyze the generation of free radical was approved by the coincidently increased of plasma MDA concentration as well as depleted of plasma GSH.

The pathology of thalassemia is, to a certain extent, associated with the generation of labile iron in the pathological RBC [23]. The appearance of such form of iron at the inner and outer cell surface exposes the cell to condition whereby the labile metal promotes self-amplifying redox reactions which simultaneously deplete cellular reducing potential (eg, GSH), catalyze the formation of ROS and accelerate RBC destruction [24].

Our study demonstrated that iron overload cause by extracellular iron administration to the mice resulting in the decreased of intracellular antioxidant GSH, increased of ROS in the RBCs and increased of peroxidative damage of RBC membrane. The overall changes of extracellular environment and intracellular condition of RBC lead to shortening of RBC life.

When administered to β-thalassemic mice, GTE show protective effects to RBCs demonstrated by several oxidant-antioxidant parameters investigated. The results indicated that GTE plays an important role as an erythrocyte antioxidant on different levels. Iron chelating activity of GTE primarily prevented an iron overload in the mice and consequently depleted the iron-induced oxidative stress. GTE also shown intracellular protective antioxidant properties in the RBCs by induced increased of intracellular GSH content, decreased level of ROS and decreased lipid peroxidation of RBC membrane. Their synergistic properties protect cells form hemolysis and thereby prolong red cell survival.

The results obtained herein allowed concluding that besides acting as antioxidants, catechins in GTE can also effectively show the iron chelating properties *in vivo*. These substances neutralize, at least in part, some of the damage that has been caused by the iron-induced production of reactive oxygen radicals. The benefit such as less toxicity and the plasma levels achievable by orally ingestion are support the therapeutic potential of green tea to be used as alternative medicine for such iron overload diseases. Since the major pathophysiology of β-thalassemia is

related to membrane damage, green tea probably useful in protecting the RBC form oxidative stress, prolonging RBC lifespan and eventually ameliorating anemic progression.

However, despite their apparent complimentary effects on erythrocytes were observed, GTE have not yet been shown to effectively ameliorate the anemia of thalassemia. GTE may be more effective if used in combination treatment with iron chelators to neutralize the deleterious effects of iron overload and oxidative stress in RBCs.

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**LIST OF FIGURE**

**Figure 1** Plasma NTBI concentrations of FE diet-fed WT (top) and BKO (bottom) mice following intervention with placebo (300 ml/kg), GTE (300 mg/kg) and DFP (50 mg/kg). Data are expressed as mean±SD (n = 12).

**Figure 2** Plasma MDA concentration of FE diet-fed WT (top) and BKO (bottom) mice following intervention with placebo, GTE (300 mg/kg) and DFP (50 mg/kg) for 3 months. Data are expressed as mean±SD (n = 12).

**Figure 3** Plasma total glutathione concentrations of of FE diet-fed WT and BKO mice following intervention with placebo (300 ml/kg), GTE (300 mg/kg) and DFP (50 mg/kg) for 3 months. Representative data at the day termination (6 months iron-loading with 3 months intervention) are are expressed as mean±SD (n = 12). $^{a}P$ <0.05 compared to WT-FE; $^{a}P$<0.05 compared to BKO-Control; $^{b}P$ <0.05 compared to BKO-FE.

**Figure 4** Blood hemoglobin concentrations of FE diet-fed WT (top) and BKO (bottom) mice following intervention with placebo (300 ml/kg), GTE (300 mg/kg) and DFP (50 mg/kg). Data are expressed as mean±SD (n = 12).

**Figure 5** Erythrocyte ROS formation of FE diet-fed WT (top) and BKO (bottom) mice following intervention with placebo (300 ml/kg), GTE (300 mg/kg) and DFP (50 mg/kg). Data are expressed as mean±SD (n = 12).

**Figure 6** Erythrocyte MDA concentration of FE diet-fed WT (top) and BKO (bottom) mice following intervention with placebo (300 ml/kg), GTE (300 mg/kg) and DFP (50 mg/kg). Representative data at the day termination (6 months iron-loading with 3 months intervention) are expressed as mean±SD (n = 12). $^{*}P$<0.05 compared to WT-Control; $^{a}P$ <0.05 compared to WT-FE; $^{*}P$ <0.05 compared to BKO-Control; $^{b}P$<0.05 compared to BKO-FE.

**Figure 7** Erythrocyte total glutathione concentrations of of FE diet-fed WT and BKO mice following intervention with placebo (300 ml/kg), GTE (300 mg/kg) and DFP (50 mg/kg) for 3 months. Representative data at the day termination (6 months iron-loading with 3 months intervention) are expressed as mean±SD (n = 12). $^{*}P$<0.05 compared to WT-Control; $^{a}P$ <0.05 compared to WT-FE; $^{*}P$ <0.05 compared to BKO-Control; $^{b}P$<0.05 compared to BKO-FE.

**Figure 8** Reduced RBC survival in thalassemic mice (BKO mice) compared to WT mice. At day = 0, murine RBCs were biotinylated by tail vein injection of sulfo-NHS-biotin. Blood was drawn at indicated time intervals. Biotinylated RBCs were labeled with phycoerythrin-conjugated streptavidin and quantified by flow cytometry. *Open circles*, values from single WT control mice; *closed circles*, averaged values from three of WT

control mice used for the curve fit; *open squares*; values from single BKO control mice; *closed square*, average values from three of BKO control mice.

**Figure 9** Red blood cells survival half-life of FE diet-fed WT and BKO mice following intervention with placebo (300 ml/kg), GTE (300 mg/kg) and DFP (50 mg/kg) for 3 months. Values are expressed as mean±SD (n = 3). $^{*}P<0.05$ compared to WT-Control; $^{\#}P<0.05$ compared to WT-FE; $^{a}P<0.05$ compared to BKO-Control; $^{b}P<0.05$ compared to BKO-FE; $^{\S}P<0.05$ compared to BKO-FE/GTE.

**Figure 10** *In vitro* protective effect of 1.25 μg GTE or DFP against iron and $H_2O_2$ induced hemolysis of red cells compared to negative control (PBS) and positive control (Trolox). Percentage of hemolysis was calculated by taking hemolysis occurred in negative control as 100%. Values are expressed as mean±SD of 6 independent experiments. $^{*}P<0.05$ compared to WT-PBS; $^{\#}P<0.05$ compared to BKO-PBS; $^{\dagger}P<0.05$ compared to BKO-GTE.

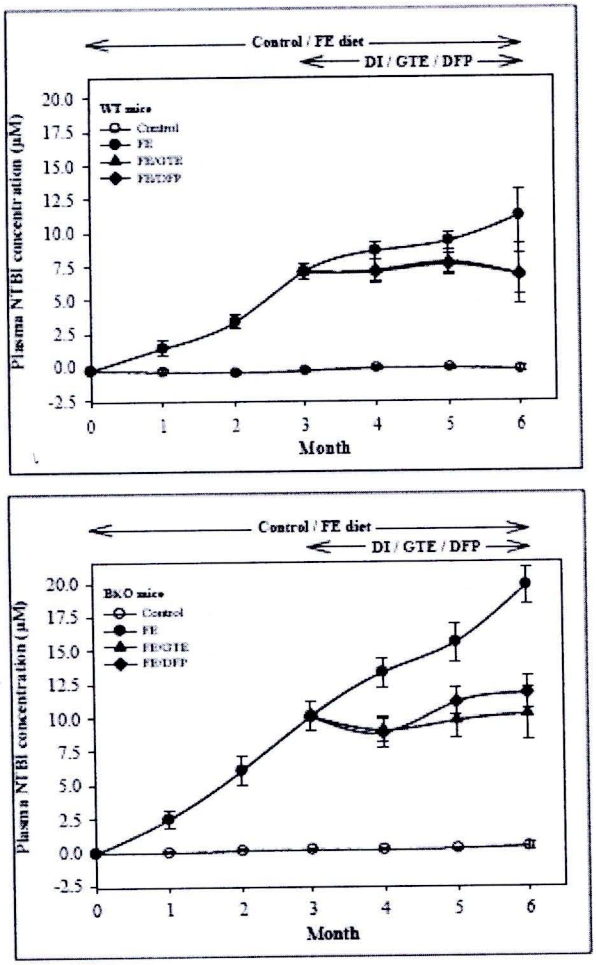


**Figure 1** Plasma NTBI concentrations of FE diet-fed WT (top) and BKO (bottom) mice following intervention with placebo (300 ml/kg), GTE (300 mg/kg) and DFP (50 mg/kg). Data are expressed as mean±SD (n = 12).

16

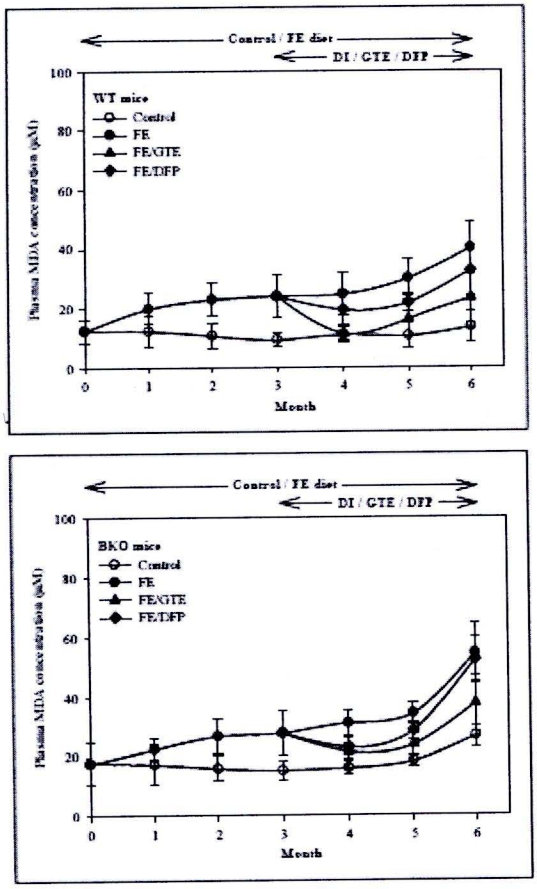


Figure 2 Plasma MDA concentration of FE diet-fed WT (top) and BKO (bottom) mice following intervention with placebo, GTE (300 mg/kg) and DFP (50 mg/kg) for 3 months. Data are expressed as mean±SD (n = 12).

17

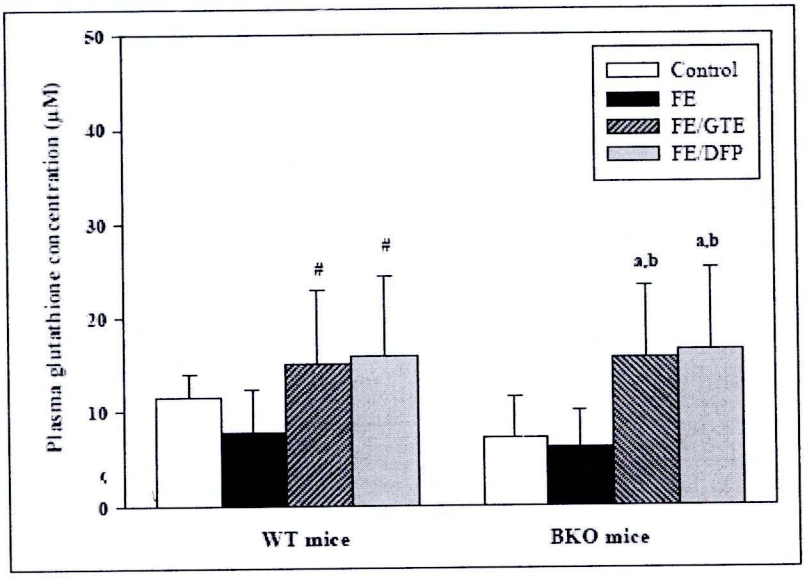


**Figure 3** Plasma total glutathione concentrations of of FE diet-fed WT and BKO mice following intervention with placebo (300 ml/kg), GTE (300 mg/kg) and DFP (50 mg/kg) for 3 months. Representative data at the day termination (6 months iron-loading with 3 months intervention) are are expressed as mean±SD (n = 12). $^{\#}p < 0.05$ compared to WT-FE; $^{a}p<0.05$ compared to BKO-Control; $^{b}p < 0.05$ compared to BKO-FE.

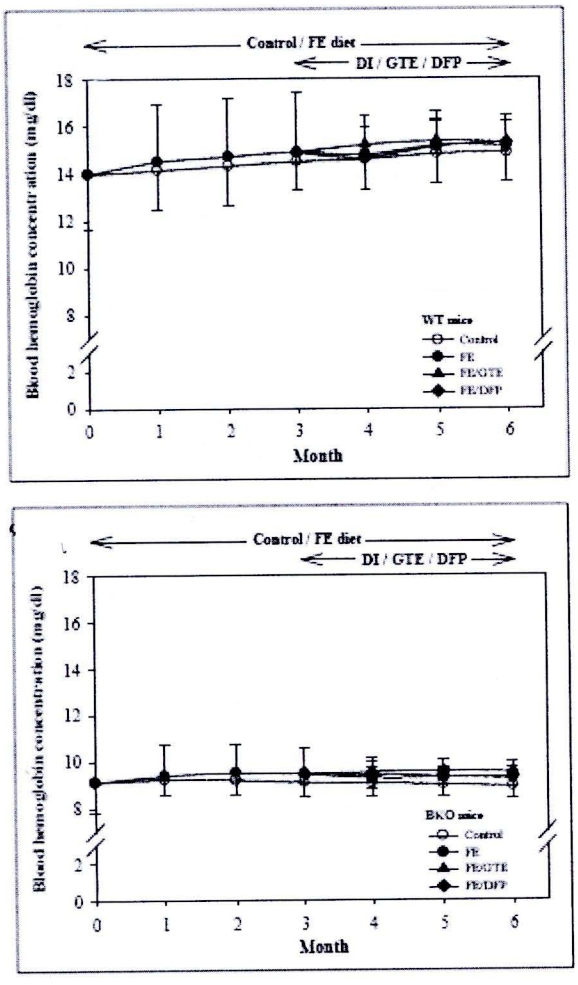


Figure 4 Blood hemoglobin concentrations of FE diet-fed WT (top) and BKO (bottom) mice following intervention with placebo (300 ml/kg), GTE (300 mg/kg) and DFP (50 mg/kg). Data are expressed as mean±SD (n = 12).

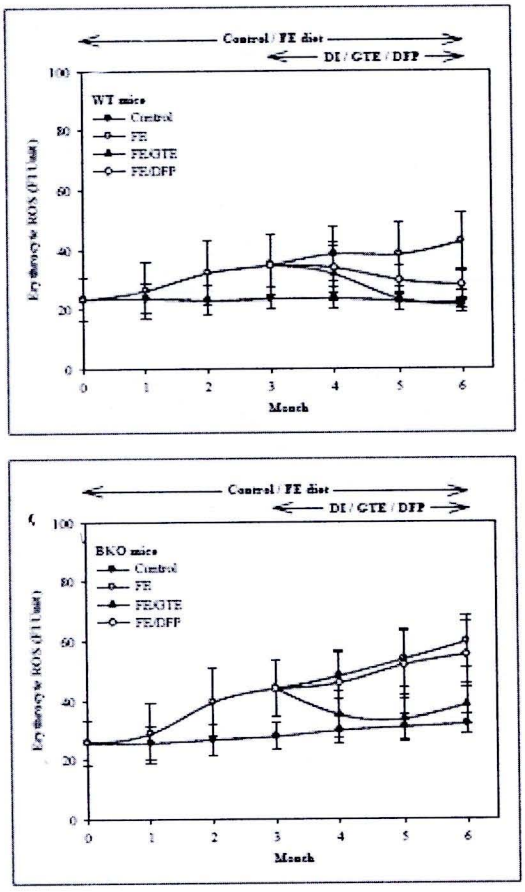


**Figure 5** Erythrocyte ROS formation of FE diet-fed WT (top) and BKO (bottom) mice following intervention with placebo (300 ml/kg), GTE (300 mg/kg) and DFP (50 mg/kg). Data are expressed as mean±SD (n = 12).

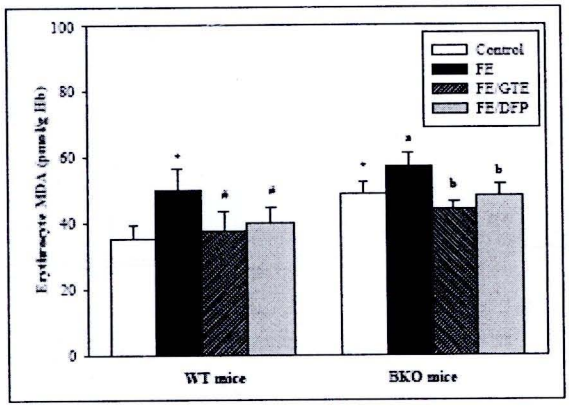


**Figure 6** Erythrocyte MDA concentration of FE diet-fed WT (top) and BKO (bottom) mice following intervention with placebo (300 ml/kg), GTE (300 mg/kg) and DFP (50 mg/kg). Representative data at the day termination (6 months iron-loading with 3 months intervention) are expressed as mean±SD (n = 12). $^{*}P<0.05$ compared to WT-Control; $^{a}P<0.05$ compared to WT-FE; $^{*}P<0.05$ compared to BKO-Control; $^{b}P<0.05$ compared to BKO-FE.

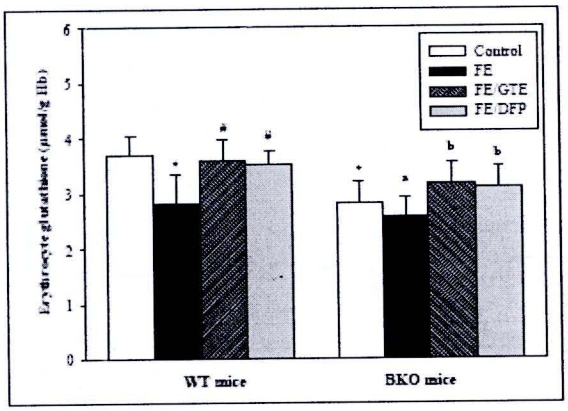


**Figure 7** Erythrocyte total glutathione concentrations of of FE diet-fed WT and BKO mice following intervention with placebo (300 ml/kg), GTE (300 mg/kg) and DFP (50 mg/kg) for 3 months. Representative data at the day termination (6 months iron-loading with 3 months intervention) are expressed as mean±SD (n = 12). $^{*}P<0.05$ compared to WT-Control; $^{a}P<0.05$ compared to WT-FE; $^{*}P<0.05$ compared to BKO-Control; $^{b}P<0.05$ compared to BKO-FE.

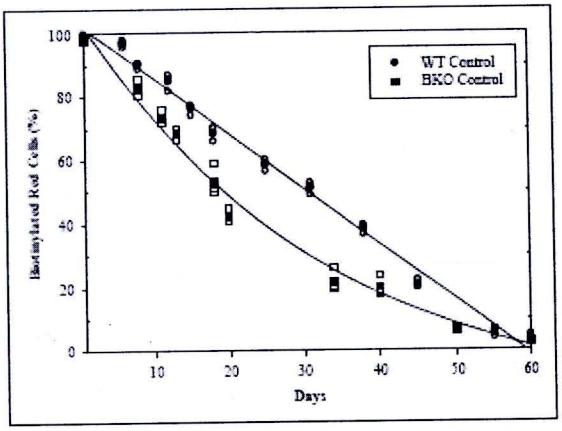


**Figure 8** Reduced RBC survival in thalassemic mice (BKO mice) compared to WT mice. At day = 0, murine RBCs were biotinylated by tail vein injection of sulfo-NHS-biotin. Blood was drawn at indicated time intervals. Biotinylated RBCs were labeled with phycoerythrin-conjugated streptavidin and quantified by flow cytometry. *Open circles*, values from single WT control mice; *closed circles*, averaged values from three of WT control mice used for the curve fit; *open squares*, values from single BKO control mice; *closed square*, average values from three of BKO control mice.

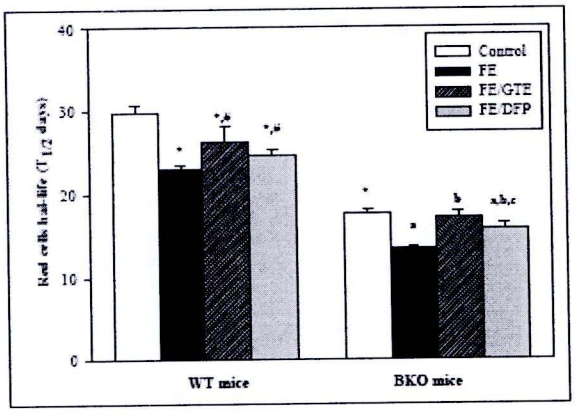


**Figure 9** Red blood cells survival half-life of FE diet-fed WT and BKO mice following intervention with placebo (300 ml/kg), GTE (300 mg/kg) and DFP (50 mg/kg) for 3 months. Values are expressed as mean±SD (n = 3). *$P$<0.05 compared to WT-Control; $^{a}P$ <0.05 compared to WT-FE; $^{b}P$ <0.05 compared to BKO-Control; $^{b}P$<0.05 compared to BKO-FE; $^{c}P$ <0.05 compared to BKO-FE/GTE.

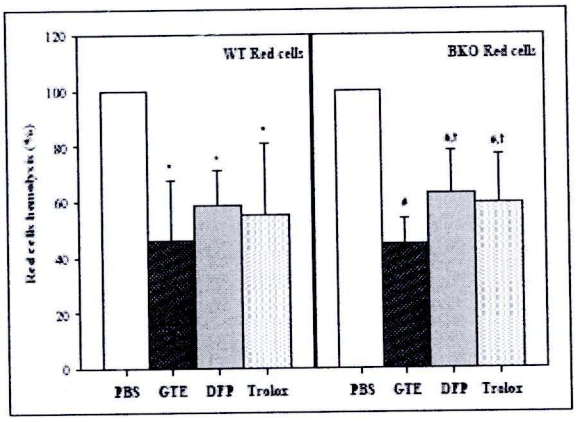


**Figure 10** *In vitro* protective effect of 1.25 μg/ml GTE or DFP against iron and $H_2O_2$ induced hemolysis of red cells compared to negative control (PBS) and positive control (Trolox). Percentage of hemolysis was calculated by taking hemolysis occurred in negative control as 100%. Values are expressed as mean±SD of 6 independent experiments. *$P$<0.05 compared to WT-PBS; #$P$ <0.05 compared to BKO-PBS; †$P$ <0.05 compared to BKO-GTE.

25

**Manuscript 2**

## Green Tea Extract and Epigallocatechin 3-gallate Reduced Labile Iron Pool and Protected Oxidative Stress in Iron-Loaded Cultured Hepatocytes

S. Srichairatanakool, K. Kulprachakarn, K. Pangit, K. Pattanapanyasat, J.B. Porter, S. Fuchaeron

**ORIGINAL ARTICLE**

**Green Tea Extract and Epigallocatechin 3-gallate Reduced Labile Iron Pool and Protected Oxidative Stress in Iron-Loaded Cultured Hepatocytes**

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**ABSTRACT**

β-thalassemia patients suffer from secondary iron overload and therefore require effective iron chelation with desferrioxamine (DFO), deferiprone (DFP) and deferasirox (DFX). Cellular and mitochondrial damage can be caused by labile iron pool (LIP) and be mediated by reactive oxygen species (ROS). Liver cells of the thalassemias have greater levels of LIP and ROS than those of healthy persons. Green tea extract (GTE) and epigallocatechin 3-gallate (EGCG) can protect human beings from cancer, hypertension, diabetes and aging. Importantly, GTE and EGCG exhibit anti-oxidative and iron-chelating activities in vitro and in vivo. We studied effects of GTE and EGCG on levels of both intracellular LIP and ROS, as well as the mitochondrial membrane potential ($\Delta\Psi$) in mouse hepatocyte and human hepatoma (HepG2) cell cultures. Calcein-AM, 2',7'-dichlorodihydrofluorescein diacetate (DCFHDA) and dihydrorhodamine 123 (DHR123) were used as specific fluorescent probes for the detection of LIP, ROS and membrane potential, respectively. Treatment with GTE and EGCG efficiently reduced levels of LIP and ROS in a concentration-dependent manner. Interestingly, these two compounds tended to increase viability of the normal hepatocytes while decreasing viability of the HepG2 cells. These results suggest that GTE and EGCG would remove the cytosolic LIP and ROS, but would not be harmful to the cells and mitochondria. Their actions might be related to iron chelation in iron-overloaded cells and iron

depletion in cancer cells. Whether these effects can improve iron overload and oxidative stress in thalassemia patients remains to be seen upon further examination.

Keywords: Green tea, epigallocatechin gallate, hepatocytes, labile iron pool, iron, reactive oxygen species.

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## INTRODUCTION

Iron is essential for many biological reactions, however, excess iron catalyzes the production of the reactive oxygen species (ROS), particularly a highly toxic hydroxyl radical via Fenton-Haber–Weiss reaction (Lesnefsky 1994). ROS are known to induce oxidation of proteins, lipids and lipoproteins, nucleic acids, carbohydrates as well as other cellular components (Britton, Ramm et al. 1994; Emerit, Beaumont et al. 2001). Accordingly, deleterious effects include cell and organelle damage, cell death, tissue necrosis, and organ dysfunction. Physiologically, transferrin-bound iron (TBI) in plasma is mainly taken up into cells via ATP-dependent transferrin receptor (TFR)-mediated endocytosis . In the event of iron overload, plasma non-transferrin bound iron (NTBI) can be taken up by facilitate diffusion, possibly via divalent metal transporter 1 (DMT1), stimulator of iron transport (SFT), zinc transport protein (Zip14) and calcium channels (Barisani, Berg et al. 1995; Parkes, Randell et al. 1995; Graham, Morgan et al. 1998; Andrews 1999; Schaefer-Mojdehkar, Zimmermann et al. 1999; Conrad and Umbreit 2000; Fleming, Migas et al. 2000; Chua, Olynyk et al. 2004; Liuzzi, Aydemir et al. 2006). Iron could function as a priming or sensitizing factor to modulate the biochemical and toxicologic actions of microsomal cytochrome P450 2E1 (CYP2E1) in rat hepatocytes and HepG2 cells (Cederbaum 2003). A previous study demonstrated that cultured hepatocytes took up TBI at very low levels. However, NTBI took up low-molecular-weight ferric ammonium citrate at high levels (Richardson, Chua et al. 1999). The release of iron from the cells was facilitated by apotransferrin and DFO (Hirsh, Konijn et al. 2002).

An intracellular accessible iron called transient iron or labile iron pool (LIP) constitutes a crossroads of metabolic pathways of iron-containing compounds and is midway between the cellular iron requirement and the hazard of ROS production. It has been postulated the LIP would be redox-active, have low-molecular-mass, and be considered a weakly chelated iron ($Fe^{2+}$ rather than $Fe^{3+}$). A wide range of LIP concentrations (3.5-230 μM) has been reported from different assays (Petrat, de Groot et al. 2002). LIP concentrations have been reported 0.9-

2.1 μM in erythroid and myeloid cells (Epsztejn, Kakhlon et al. 1997), 0.17±0.85 μM in peripheral blood lymphocytes (Gackowski, Kruszewsk et al. 2002), 0.18-0.57 μM in mouse lymphoma cells (Lipinski, Drapier et al. 2000), 5.0±2.0 μM in rat hepatocytes, 6.6±2.9 μM in rat hepatocyte nucleus, 11.8±3.9 μM in rat liver endothelial cell nuclei, 4.8±2.3 μM in mitochondria of rat hepatocytes, 9.2±2.7 μM in mitochondria of rat liver endothelial cells and 15.8±4.1 μM in lysosomal/endosomal compartments (Petrat, de Groot et al. 2001). LIP plays a role in the induction of cellular and tissue oxidative damage. High LIP content can cause DNA damage in the nucleus and is positively correlated with DNA damage in mouse lymphoma cells L5178Y and human lymphocytes.

Liver is the main body site for iron stores and homeostasis. Under liver iron overload and oxidative stress, free radicals and membrane oxidation by-products can trigger liver organelle dysfunction, inflammation, fibrosis, and eventually cell death (Corradini, Ferrara et al. 2004). Treatment of LIP with iron chelators or antioxidants prevents nuclear and mitochondrial DNA breaks of the liver cells. Uptake of DFO into hepatocytes was several hundred-fold times faster than the uptake of DFO into red cells (Porter, Rafique et al. 2005). The formation of pores or leaks in the mitochondrial membrane possibly occurred through the activation or dimerization of pro-apoptotic proteins including Bax, activated Bid, Bak and Bad. The activation or dimerization of such proteins, the collapse of electrochemical gradient or potentials ($\Delta\Psi$) across mitochondrial membrane, the release of cytochrome c into cytoplasm, and the activation of caspases are responsible for cell apoptosis. An association of CYP2E1-dependent oxidative stress, mitochondrial membrane-potential collapse, and GSH homeostasis possibly contributes toward the developing toxicity of iron to the liver (Caro and Cederbaum 2004).

The extract of leaves of *Pistacia lentiscus* exerted antioxidant activity in HepG2 cell culture but had a hepatotoxic effect in rats (Ljubuncic, Azaizeh et al. 2005). EGCG of green tea protected HepG2 cells from CYP2E1-dependent oxidative stress and toxicity (Jimenez-Lopez and Cederbaum 2004). The treatment of antioxidants (such as N-acetyl cysteine and vitamin E) together with iron chelator could neutralize deleterious effect of oxidative stress in thalassemia patients with iron overload (Rachmilewitz, Weizer-Stern et al. 2005). Green tea (*Camellia sinensis*) contains at least five catechin derivatives including gallic acid (GA), epigallocatechin (EGC), epicatechin (EC), epigallocatechin 3-gallate (EGCG), and epicatechin 3-gallate (ECG), of which EGCG is a major active ingredient (Khokhar, Veeneman et al. 1997; Srichairatanakool, Ounjaijean et al. 2006). Our recent studies have shown that green tea reduced iron overload and oxidative stress in rats (Ounjaijean, Thephinlap et al. 2007) and inhibited the accumulation of iron in the livers of wild-type and thalassemic mice (Saewong, Ounjaijean et al. 2010). Recently, green tea catechins have been shown to

counteract oxidative mitochondrial damage in the livers of reserpine-treated rats (Al-Bltoushi, Safer et al. 2009). However, the order of cytotoxicity of tea catechins derivatives to primary rat hepatocytes has been reported EGCG=ECG>GA, EGC>EC (Galati, Lin et al. 2006). Some antioxidant compounds such as curcumin and quercetin induced apoptosis in human HepG2 cells through mitochondrial DNA damage and hyperpolarization (Cao, Liu et al. 2007; Chang, Hsu et al. 2009). The combination of chloroform extract from *Ziziphus jujube* and green tea enhanced the inhibition of HepG2 cell growth (Huang, Kojima-Yuasa et al. 2008).

We studied the effects of crude extract and EGCG of green tea on levels of LIP, ROS, mitochondrial membrane potential in primary hepatocyte and HepG2 cell cultures. Cytotoxicity of the compounds to the hepatocytes was also investigated.

## MATERIALS AND METHODS

### *Chemicals and Reagents*

Acetonitrile, absolute ethanol, ethyl acetate, and methanol are the highest pure solvents. Calcein-AM solution (Invitrogen Corperation, CA,USA), dihydrorhodamine 123 (DHR123) (Biotium, Inc., CA, USA), and 2',7'-dichlorodihydrofluorescein diacetate (DCFHDA) (Sigma-Aldrich, St. Louis, MO, USA) are fluorescent probes. Collagenase type IV, Dulbecco's modified eagle medium (DMEM), Krob-Ringer buffer (KRB), penicillin-streptomycin, 0.5% trypsin-EDTA solution and fetal bovine serum were purchased from GIBCO® Invitrogen, CA, USA. Insulin (Humulin®R) is a product of Health Central Network Inc., USA. Dexamethazone, dihydrogen phosphate potassium salt ($KH_2PO_4$), disodium hydrogen phosphate ($Na_2HPO_4$), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT dye), ethylene glycol-bis(2-aminoethylether) -N,N,N',N'-tetraacetic acid (EGTA), hydrogen peroxide (30%), and epigallocatechin 3-gallate (EGCG) were obtained from Sigma-Aldrich, St Louis, MO, USA. Dimethyl sulfoxide (DMSO) (Fisher Scientific, UK), ferric ammonium citrate (FAC) (BDH, England) and Desferrioxamine mesylate (DFO) (Novartis, Switzerland) were purchased from a drug store in Maharaj Nakorn Chiang Mai Hospital, Faculty of Medicine, Chiang Mai University. Deferiprone (DFP) was kindly donated by Dr. Chada Phisalapong, Government Pharmaceutical Organization Thailand.

### *Green tea and EGCG preparations*

Fresh tea shoots were harvested and immediately dried in a microwave oven (3 minutes, 800 watts, 100°C). Dry tea leaves were extracted with hot deionized water (80°C) for 15 minutes and filtered through 0.45-μm membrane (cellulose acetate type, Millipore, Maidstone, England) and dried under a vacuum. Results of HPLC

analysis using the column (Waters SpheroSorb-ODS2, 250x4.7 mm, 5-μm), mobile-phase solvent (0.05% $H_3PO_4$ : acetonitrile : ethyl acetate = 86:12:2, v/v/v), a flow rate of 1.0 ml/min as well as the detection of 280 nm indicated amounts of EGC, C, EC, EGCG, ECG and total catechins persisting in the GTE were 102, 5.1, 53.9, 88.7, 4.3 and 255 mg/g dry weight, respectively (Srichairatanakool, Ounjaijean et al. 2006, Saewong, Ounjaijean et al. 2010).

EGCG was isolated from green tea extract (5.0%, w/v) using semi-preparative HPLC (Thaphimlap 2007) according to following conditions: main column (Luna ODS2, 250X10 mm, 5 μm, Phenomenex®, Torrance, California, USA) connected to guard column (Luna ODS2, 50x10 mm, 5 μm, Phenomenex®, Torrance, California, USA), isocratic elution with solvent (methanol:$H_2O$ = 29:71, v/v) at a flow rate of 1.0 ml/min, and detection of catechins derivatives at 280 nm. EGCG fraction was pooled and checked for purity using the analytical HPLC as described above. Methanol constituted in the pooled fractions was removed under a vacuum using a freeze-dry technique. Prior to being used, lyophilized EGCG fraction was stored in the dark at -20°C.

*Isolation and cultures of hepatocytes*

Mice (C57/BL6, 9 to 12 weeks old, 30 to 35 g) were provided by Professor Suthat Fucharoen at Thalassemia Research Center, Institute of Molecular Biosciences, Mahidol University and used as a source of primary hepatocytes. The animal study was conducted with the approval of the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Medicine, Chiang Mai University (Reference Number -3/2548).

Mice were anesthetized with vapor diethyl ether and their chests were opened. The liver was perfused *in situ* via the portal vein with the Krob-Ringer buffer (KRB), pH 7.4 comprising 116 mM NaCl, 5.4 mM KCl, 25 mM $NaHCO_3$ and 0.63 mM EGTA at 37°C, a flow rate of 1 ml/min for 20 minutes and with the KRB buffer containing 1 mM $CaCl_2$, 0.025% (w/v) collagenase type IV for 20 minutes. The livers were excised, teased apart, incubated at 37°C for a further 15 minutes in the collagenase solution, and isolated hepatocytes were harvested through nylon mesh (250-61 μm). Crude cells were sedimented by differential centrifugation (60g) for 5 minutes at 25°C and resuspended in the 20 mM HEPES buffer containing 116 mM NaCl, 5.4 mM KCl, 1 mM $CaCl_2$, pH 7.4. Cell viability was assayed using trypan blue exclusion technique. Cell numbers were adjusted to $4\times10^5$ viable cells/ml and cultured in DMEM supplemented with 10% (v/v) FBS, 2.0 mM glutamine, 100 U/ml penicillin/100 U/ml streptomycin, 200 mU/ml insulin and 1 μM dexamethasone (Schmidt, Schmitz et al. 2005).

Human hepatoma (HepG2) cells were used to study the biochemical and toxicological properties due to their increased oxidative stress, loss of mitochondrial function, and loss of viability when challenged with

proxidants such as iron (Wu and Cederbaum 2008). Cells were cultured in complete DMEM containing 2 mM glutamine, pyridoxine hydrochloride, 110 mg/l sodium pyruvate, 10 mM HEPES, 44 mM $NaHCO_3$, 10% (w/v) inactivated FBS and 0.01% (w/v) penicillin-streptomycin 37°C (Huang, Chen et al. 2001).

***Chelation of intracellular LIP of hepatocyte cultures by GTE and EGCG***

Primary hepatocytes and HepG2 cells ($5x10^4$cells/well) were exposed to FAC solution (0.5 mM) at 37°C under 5% $CO_2$ atmospheric conditions for 24 hours and washed three times with PBS solution, pH 7.4 to remove the excess iron (Trinder, Bassy et al. 1990; McAbee and Ling 1997). Solutions of GTE (0-100 mg/dl), EGCG (0-200 μM), DFP (0-200 μM), DFO (0-200 μM), GTE (0-100 mg/dl) with 25 μM DFP and EGCG (0-200 μM) with 25 μM DFP were freshly prepared in 50 mM HEPES buffer, pH 7.2 and filtered through a membrane (cellulose type 0.22 μm). The cells were incubated with the GTE, EGCG, DFP, DFO, GTE plus DFP and EGCG plus DFP solutions at 37°C for 6, 12 and 24 hours (Staubli and Bookstaft 1998). The treated cells were washed three times with the culture medium and labeled with calcein-AM solution (50 μM in DMSO). Fluorescent intensity (FI), which was inversely proportional to the analyzed amount of LIP, was measured with a 96-well plate reader spectrofluorometer (absorption/excitation wavelengths 485 nm/530 nm) (Epsztejn, Kakhlon et al. 1997). Viability of studied cells was greater than 80% and was not changed during the assay.

***Reduction of ROS levels of cultured hepatocytes by GTE and EGCG***

DCFH-DA can simply diffuse into the cells and be hydrolyzed by esterase in viable cells to produce 2',7'-dichlorofluorescein (DCFH), which will be subsequently oxidized by existing ROS to 2',7'-dichlorofluorescein (DCF). An increase of a green fluorescent signal indicates increased intracellular oxidative stress. The hepatocytes were incubated with the compounds as above at 37°C for 0, 6, 12 and 24 hours. The treated cells were washed three times with the culture medium, labeled with DCFH-DA solution (10 μM in methanol) for 30 minutes, and challenged with $H_2O_2$ solution (125 μM). FI was measured using the spectrofluorometric technique (excitation/emission wavelengths 485 nm/530 nm) (Perez-de-Arce, Fences et al. 2005).

***Effects of GTE and EGCG on mitochondrial membrane potentials of cultured hepatocytes***

A cationic probe dihydrorhodamine 123 (DHR123) is used to signal the loss of mitochondrial membrane potential which results in changes in the fluorescent intensity termed redistribution signals. Nonfluorescent DHR123 readily enters most of the cells into the mitochondrial matrix in response to mitochondrial membrane potential and can be oxidized by cellular ROS or redox system to the fluorescent R123 that accumulates in

mitochondrial membranes (Petit, Izbicka et al. 1999). Briefly, the hepatocytes treated with the compound(s) as above were centrifuged at 1000g, 4°C for 1 minute. Cell pellet was resuspended in 2 ml of the medium containing 1.5 uM DHR123, incubated under 5% $CO_2$ atmospheric conditions at 37°C for 10 minutes. After incubation, the cells were separated by centrifugation at 1000g, 4°C for 1 minute and FI was measured using the spectrofluorometric technique (excitation/emission wavelengths 490 nm/520 nm). The capacity of mitochondria to take up the R123 was calculated as the difference of FI between untreated cells (control) and treated cells (Galati, Lin et al. 2006).

### Statistical Analysis

The data were expressed as mean ± standard deviation (SD) of measurements. The statistical difference of analyzed data was determined by using the Student's *t*-test or ANOVA. A comparison between groups was done using the non-parametric Mann-Whitney U test. $p < 0.05$ was considered significant difference.

## RESULTS AND DISCUSSION

Normally, iron is essential for many biochemical functions and metabolic pathways of human life and higher vertebrates. Pathophysiologically, iron imbalance can cause anemia and oxidative stress. Diseases that are related to or associated with iron overload can be exemplified by hereditary hemochromatosis, β-thalassemia, fibrosis, cirrhosis, and cancer. Oxidative stress is a consequence from overproduction of iron-catalyzed free radicals via Haber-Weiss and Fenton reactions. This can deteriorate the functions of several vital organs such as heart, liver, kidneys, brain and pancreas. Liver is the main organ for iron storage and metabolism, which can be affected by excessive redox iron and reactive oxidants. Drug regimens such as iron-chelator or antioxidant are recommended for these patients to relieve oxidative tissue damage and improve their quality of life (Olivieri, Brittenham et al. 1995; Porter 1997). So far, DFO, DFP and DFX have been world widely used for treatment of thalassemia patients with iron overload. Their side effects are associated and carefully monitored (Porter 1997; Britton, Leicester et al. 2002). Importantly, the development of an economical and orally effective iron chelator is of great importance (Porter 2009). Iron-overload diseases frequently develop hepatocellular carcinoma, which the pathogenesis might involve an oxidative process via the intermediate production of reactive oxygen species.

Phytochemicals possess outstanding antioxidant and free radical-scavenging properties, suggesting a possible protective role in man (Scott, Butler et al. 1993). Green tea (GT) is an excellent source of antioxidative polyphenols including catechin, epicatechin, epigallocatechin, epigallocatechin gallate, epicatechin gallate, and

gallocatechin gallate. The beneficial effects of green tea catechins would be applicable for treatment of β-thalassemia major patients.

Examination of the hepatocellular iron content of liver specimen is required for the diagnosis of iron overload (Valberg, Ghent et al. 1978). D-tagatose, an antioxidant and weak iron chelator, removed redox-active iron and decreased oxidative stress in primary cultures of hepatocytes derived from male C57BL/6 mice (Paterna, Boess et al. 1998).

Under the iron overload, non-specific iron in called NTBI or LIP is transported in transferrin-saturated plasma and rapidly cleared by the liver. An in vitro study reported that rat hepatocytes in primary culture had a high capacity to take up the NTBI in forms of ferric citrate and small-molecular-weight iron complexes by facilitated diffusion (Baker, Baker et al. 1998; Richardson, Chua et al. 1999).

Iron uptake into the cultured hepatocytes of wild type mice were less than the cultured HepG2 cells because HepG2 cells as a human hepatoma cell line. It used iron for growth and proliferation more than normal cells. Especially, at 6 hours it was found that the iron uptake of treated-hepatocytes and HepG2 cells cultured were decreased. Primary hepatocytes are widely used as a cell culture model to identify mechanisms of hepatocellular damage, though there are some examples for compounds causing hepatic damage *in vivo* which could not be demonstrated in liver cells *in vitro*.

Our study has shown that GTE and EGCG decreased concentrations of chelatable, transients irons implied as LIP in both primary hepatocytes and HepG2 cells cultures in dose-dependent manner. Interestingly, the natural products cooperated with DFP to remove the persisting LIP in the target cells. Similarly, standard iron chelators as DFP and DFO were able to lowered the LIP concentrations in the hepatocytes. It can be summarized that GTE and EGCG are potential to chelate intracellular transient iron and work together with DFP for the chelation in the liver cells with iron overload. Dose of the phytochemical compounds is important and needs optimizing to achieve effective chelation and minimal adverse effects. Gaboriau and colleagues showed that CP20 (or DFP) and CP411 efficiently protected cultured primary rat hepatocytes and rat hepatoma cells from toxic effect of iron load (Gaboriau, Chantrel-Groussard et al. 2004). One study demonstrated that DFO penetrate into the hepatocytes and chelated cytosolic LIP slowly whereas DFP and DFX readily entered the cells and efficiently chelated the LIP (Glickstein, El et al. 2005). Synthetic alpha-ketohydroxypyridine chelators such as 1,2-dimethyl (DFP) and 1-ethyl-2-methyl derivatives of 3-hydroxypyrid-4-one were active in removing iron from reticuloendothelial system and hepatocytes, and superior to DFO (Brock, Licenga et al. 1990).

GTE and EGCG immediately reduced levels of ROS in the cultured primary hepatocytes as well as HepG2 cells in dose-dependent manner. DFP and DFO did not change any levels of the ROS persisting in the cells. Suggestively, green tea catechins, particularly EGCG exhibit not only antioxidative but also iron-chelating properties, which can penetrate into the cells and interact with intracellular reactive oxidants including redox-active iron and free radicals. Since HepG2 cells are highly metabolic human hepatoma cells and produce larger amounts of reactive oxidants, their free-radical scavenging activity seems to be more apparent than that of primary hepatocytes. EGCG was postulated to be a potent antioxidant and effectively afforded protection of rat primary hepatocytes from free radicals-mediated diseases (Ramirez-Mares and de Mejia 2003). Mushroom (*Phellinus linteus*) protected primary rat hepatocytes from FeNTA-induced oxidative damage and attenuated cytotoxicity of other oxidants, possibly by increasing antioxidant (such as reduced glutathione) concentration and enhancing antioxidant enzyme activities (such as glutathione reductase, glutathione peroxidase, superoxide dismutase) (Meloche and O'Brien 1993; Ye, Hou et al. 2007).

The liver is chiefly responsible for taking up and storing excessive amounts of iron. The major hepatic toxicities of iron overload include damage to multiple cell types (hepatocytes, Kupffer cells, hepatic stellate cells) and to multiple subcellular organelles (mitochondria, lysosomes, and smooth endoplasmic reticulum) (Bonkovsky and Lambrecht 2000). GTE and EGCG were able to decrease fluorescent intensity of rhodamine 123 (R123) representing mitochondrial ROS in primary hepatocytes and HepG2 cells. DFP and DFO induced ROS formation, affected and collapsed mitochondria membrane potential, particularly at 6 hours.

An evidence showed that tea phenolic acids and catechins containing gallic acid moieties were toxic toward isolated rat hepatocytes, which the order of cytotoxicity was EGCG=propyl gallate>ECG=gallic acid, EGC=EC (Galati, Lin et al. 2006). Our study has demonstrated that GTE, EGCG, DFP and DFO were toxic to the HepG2 cells, depending on applied doses and degree of the toxicity, which was GTE>DFO>DFP>EGCG. Duration of treatment and chelation also influenced the cytotoxic effect. GTE and EGCG treatments slightly increased number of viable primary hepatocytes, it is probable that the normal primary hepatocytes utilized their antioxidant activity to prevent cellular oxidative damage and brought their nutritional constituents of growth and development. Recently, researchers have mentioned that combination of the chloroform fraction from drink tea (*Zizyphus jujuba*) and GTE enhanced inhibition of HepG2 cell growth in G1 arrest phase, suggesting synergism in anticancer activity (Huang, Kojima-Yuasa et al. 2008). Extract of herbal tea (*Ardisia compressa*) had an antioxidant protective effect on rat hepatocytes when exposed to 1-NP and was superior to EGCG anti-oxidation (de Mejia and Ramirez-Mares 2002). EGCG was best at suppressing growth, inducing apoptosis in human

prostate cancer DU145 cells and also displayed strong growth inhibitory effects against the lung tumor cell line H661.

Previous studies have shown that iron suppressed the expression of chimeric human transferrin genes in the livers of transgenic mice and regulated the synthesis of endogenous transferrin in the HepG2 cells (Barnums-Huckins and Adrian, Cell Biol Int., 2000). Iron was found to be cytotoxic in CYP 2E1-overexpressing HepG2 cells (E47 cells). Stimulation with bacterial lipopolysaccharide increased uptake of transferrin-bound iron into hepatic parenchymal cells and HepG2 cells (Hirayama et al. Hepatology, 1993). In development of toxicity by exposure of the hepatocytes to the tested compounds, the toxicity was prevented by antioxidants.

Makino (Crossostephium chinensis L.) showed antioxidant, iron-chelating and anti-proliferative activity in HepG2 cells (Chang et al. Am J Chin Med, 2009). The toxicity of increased ROS and arachidonic acid in the HepG2 cells when the cells are enriched with arachidonic acid can be prevented by antioxidants and iron chelator DFO (Chen et al. Alcohol Clin Exp Res, 1998). DFP and DFO have been shown to inhibit HepG2 cell proliferation in the S-phase of cell cycle (Chenoufi et al. Biochem Pharmacol, 1998). The ratio of DFP to iron (at least 3:1) is optimal to inhibit ROS generation; nevertheless, lower concentrations of the DFP can potentiate oxidative DNA damage in iron-loaded HepG2 cells (Cragg et al. Blood, 1998).

In conclusion, water extract of green tea (*Camellia sinensis*) and epigallocatechin 3-gallate could reduce excessive redox-active, transient (labile) iron and reactive oxygen species in cytosolic compartment of ex-vivo mouse primary hepatocytes as well as HepG2 cell cultures. The EGCG scavenges mitochondrial free radicals in the hepatocytes effectively, which the benefit would prevent mitochondrial membrane damage, hepatocyte apoptosis and liver damage. Green tea extract and EGCG are not toxic to the primary hepatocytes but toxic to the HepG2 cells when treat at high dose. The extract also enhances growth and viability of the hepatocytes, it would probably regenerate the damaged livers exposed to iron, free radicals and xenobiotics. Collectively, our findings imply protective and therapeutic effects of green tea catechins on the liver with iron overload and oxidative stress. Most importantly, it needs designing the merit adjunctive study of green tea products and deferiprone to prevent liver pathogenesis in thalassemia patients with iron overload in the near future.

ACKNOWLEDGEMENTS

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LIST OF FIGURES

**Figure 1** Levels of LIP in iron-loaded hepatocyte cultures treated with GTE, EGCG, DFP and DFO for 6, 12 and 24 hours. Data were obtained from three independent triplicate experiments and shown as mean±SD. *p<0.05 when compared to control cells; *p<0.05, **p<0.01, ***p<0.005 when compared to iron-loaded cells.

**Figure 2** Levels of LIP in iron-loaded hepatocyte cultures treated with GTE and EGCG together with 25 μM DFP for 6, 12 and 24 hours. Data were obtained from three independent triplicate experiments and shown as mean±SD. ^p<0.05, ^^p<0.01, ^^^p<0.001 when compared to treatment with 25 μM DFP alone.

**Figure 3** Levels of ROS in iron-loaded hepatocyte cultures treated with GTE, EGCG, DFP and DFO for 6, 12 and 24 hours. Data were obtained from three independent triplicate experiments and shown as mean±SD. *p<0.05 when compared to control cells; *p<0.05, **p<0.01, ***p<0.005 when compared to iron-loaded cells.

**Figure 4** Levels of ROS in iron-loaded hepatocyte cultures treated with GTE and EGCG together with 25 μM DFP for 6, 12 and 24 hours. Data were obtained from three independent triplicate experiments and shown as mean±SD. ^p<0.05, ^^p<0.01, ^^^p<0.001 when compared to treatment with 25 μM DFP alone.

**Figure 5** Levels of mitochondrial membrane potentials (mΨ) in iron-loaded hepatocyte cultures treated with GTE, EGCG, DFP and DFO for 6, 12 and 24 hours. Data were obtained from three independent triplicate experiments and shown as mean±SD. *p<0.05 when compared to control cells; *p<0.05, **p<0.01, ***p<0.005 when compared to iron-loaded cells.

**Figure 6** Levels of mitochondrial membrane potentials (mΨ) in iron-loaded hepatocyte cultures treated with GTE and EGCG together with 25 μM DFP for 6, 12 and 24 hours. Data were obtained from three independent triplicate experiments and shown as mean±SD. ^p<0.05, ^^p<0.01, ^^^p<0.001 when compared to treatment with 25 μM DFP alone.

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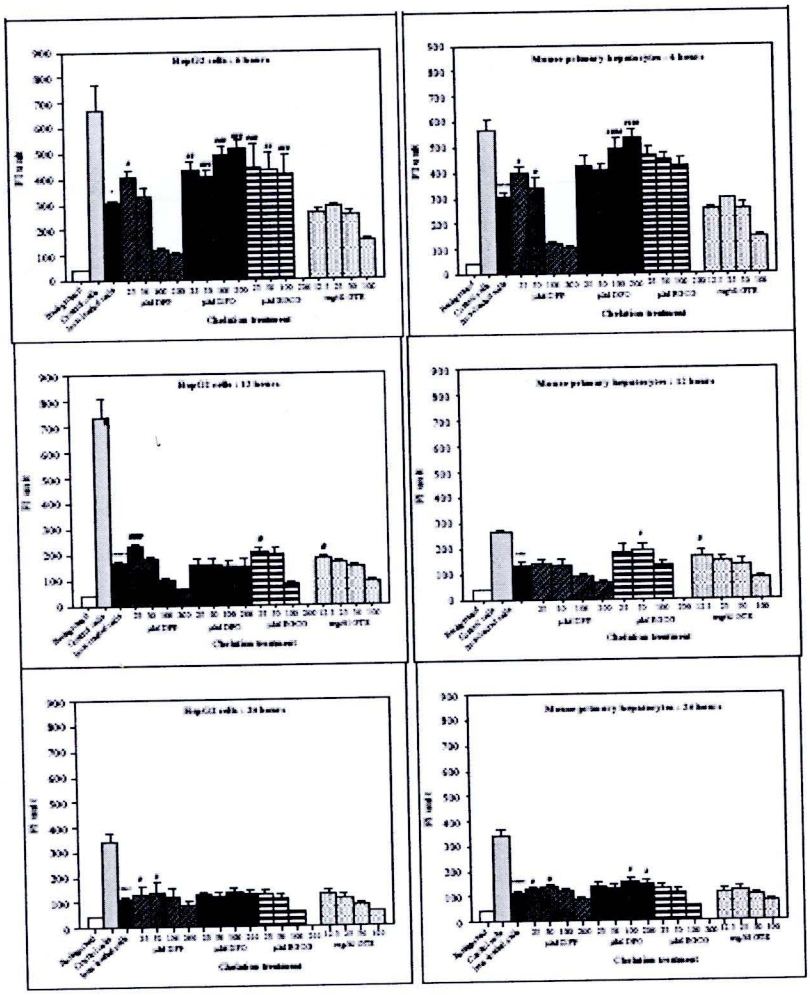
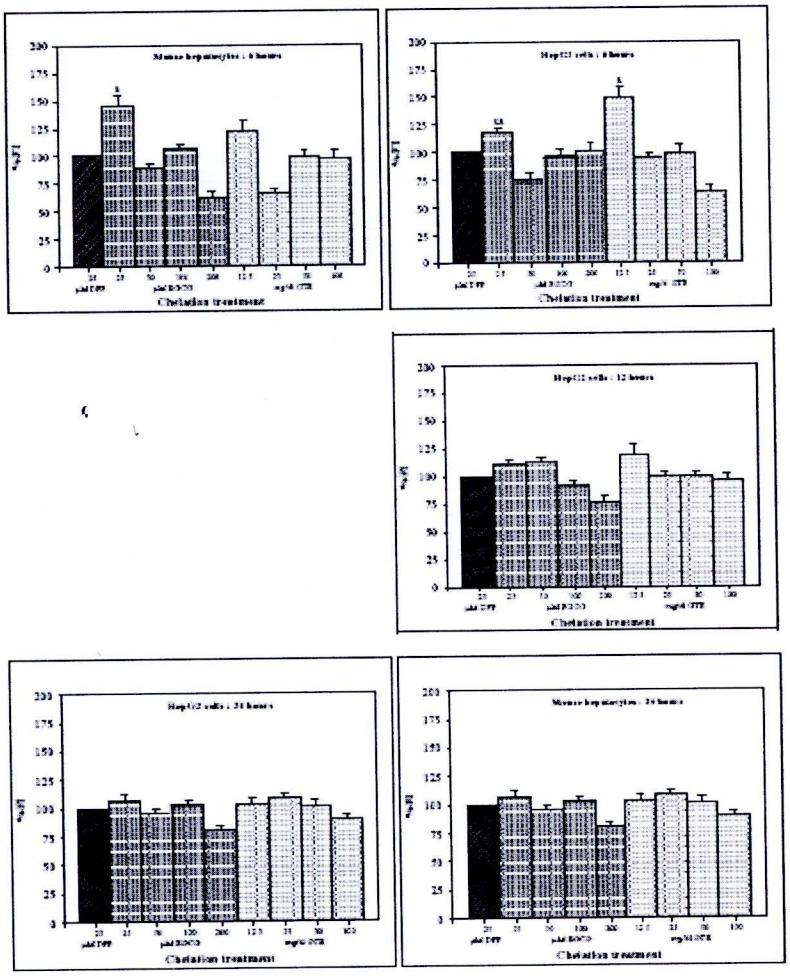


Figure 2 Levels of LIP in iron-loaded hepatocyte cultures treated with GTE and EGCG together with 25 μM DFP for 6, 12 and 24 hours. Data were obtained from three independent triplicate experiments and shown as mean±SD. $^{A}p<0.05$, $^{AA}p<0.01$, $^{AAA}p<0.001$ when compared to treatment with 25 μM DFP alone.



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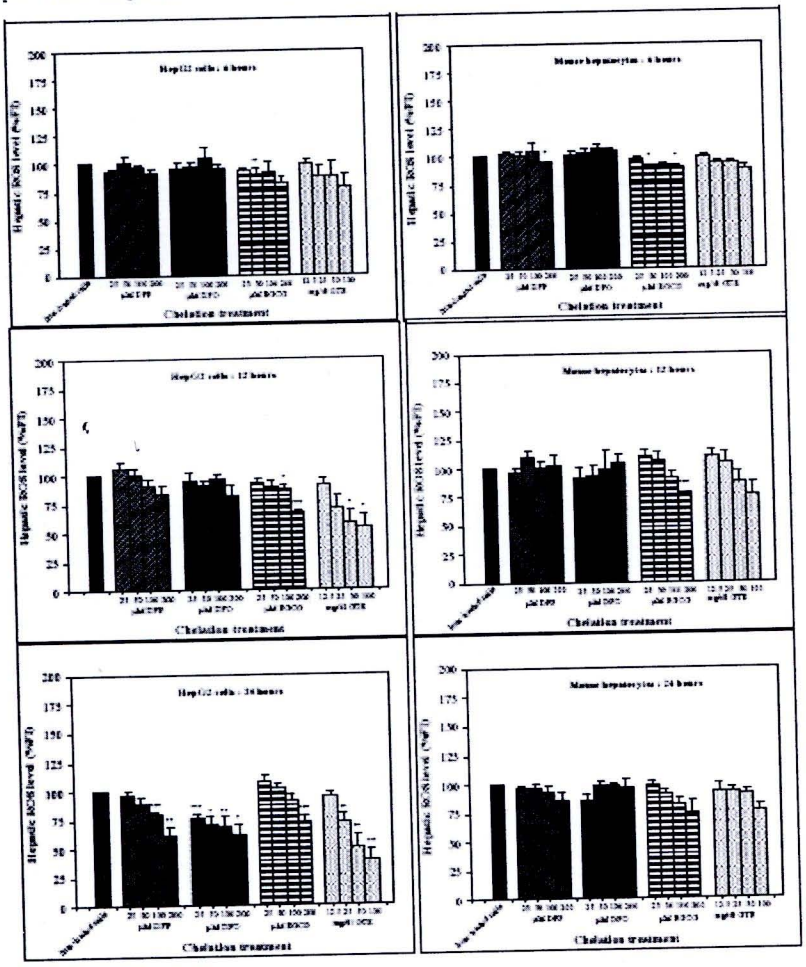

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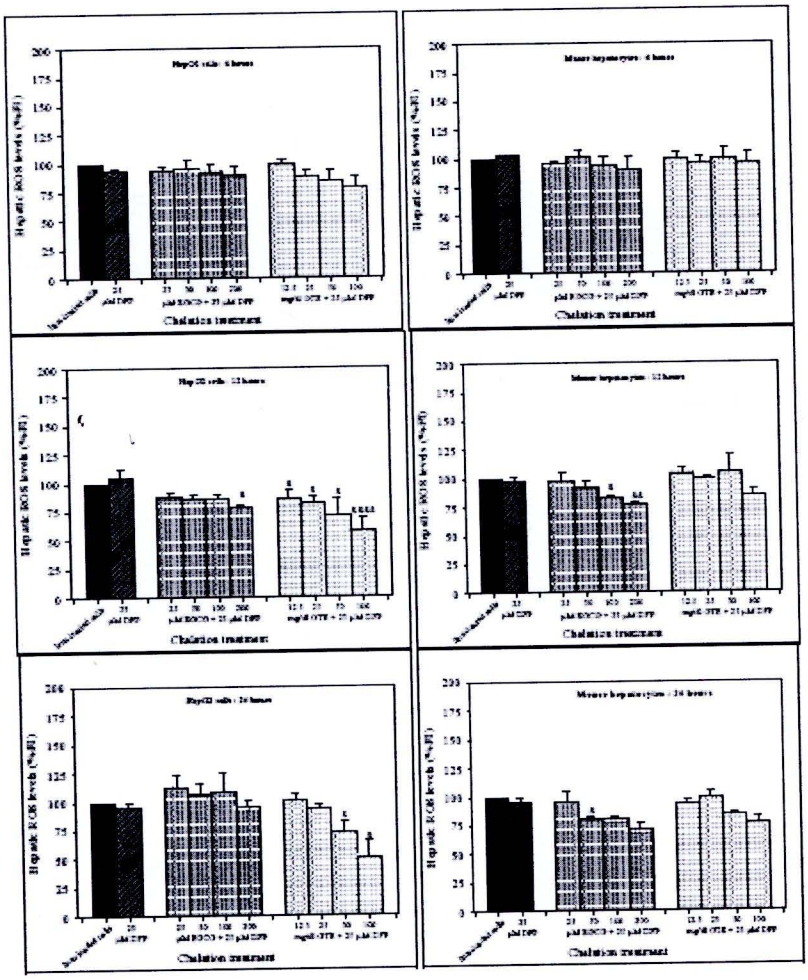
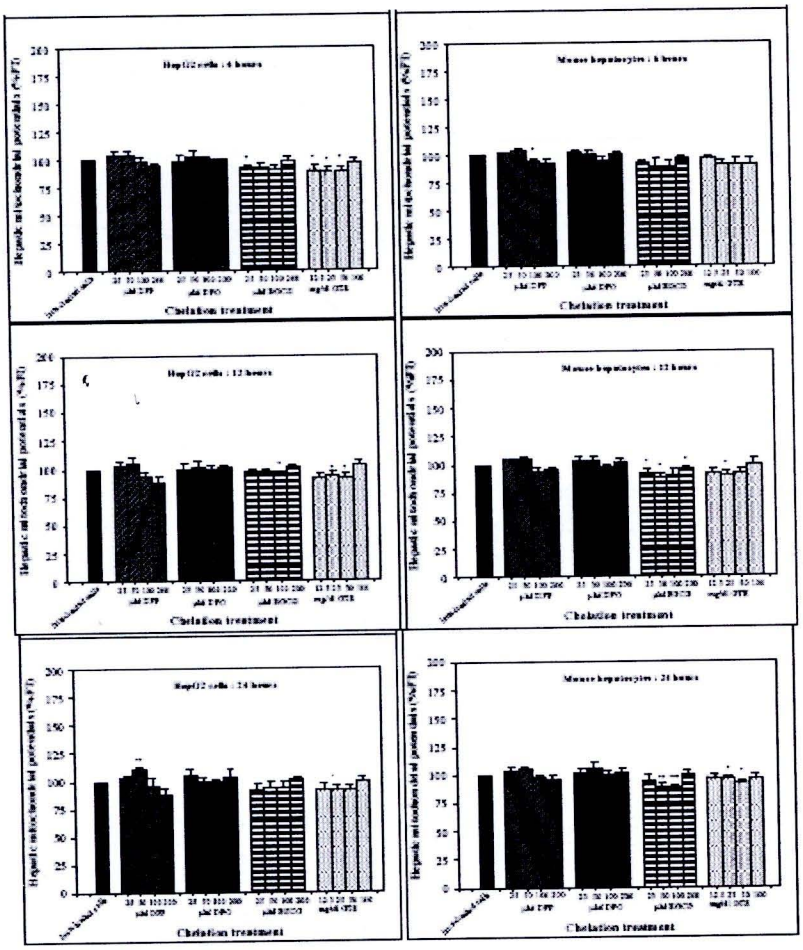


Figure 5 Levels of mitochondrial membrane potentials (mΨ) in iron-loaded hepatocyte cultures treated with GTE, EGCG, DFP and DFO for 6, 12 and 24 hours. Data were obtained from three independent triplicate experiments and shown as mean±SD. *p<0.05 when compared to control cells; *p<0.05, **p<0.01, ***p<0.005 when compared to iron-loaded cells.



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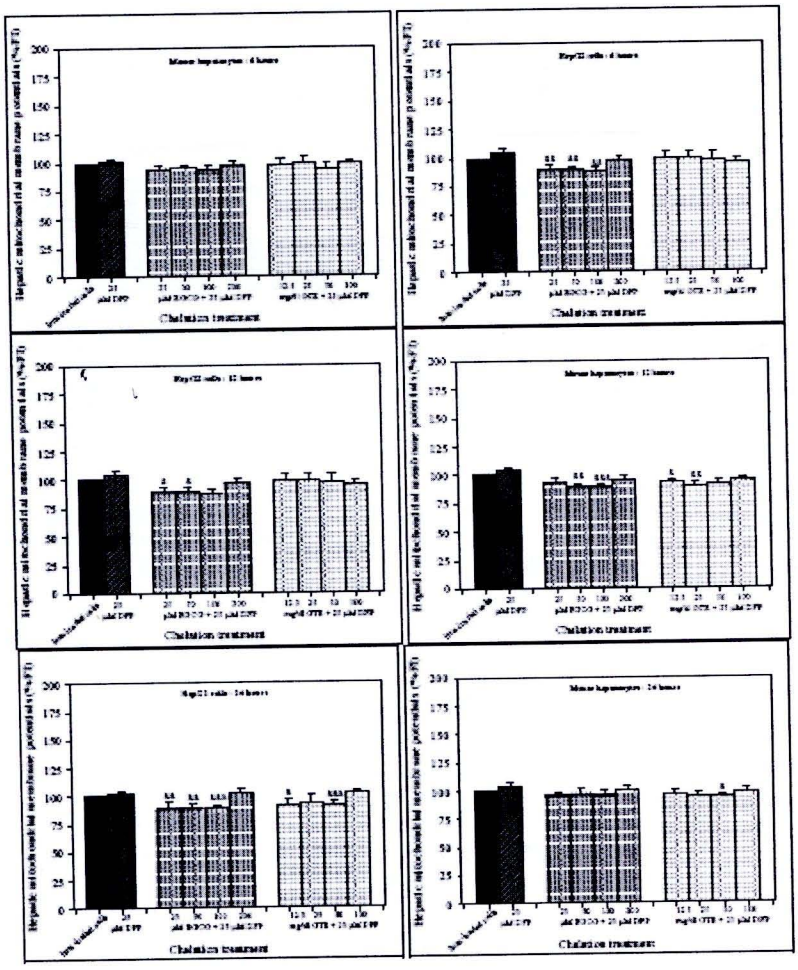

**Conference Abstract 1**

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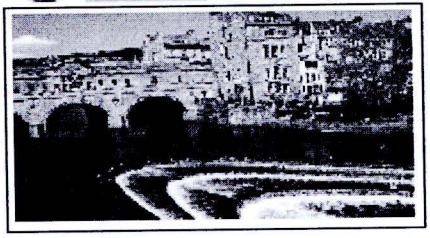

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Prof Robert Evans , Brunel University, UK
Email: robertevans49@yahoo.co.uk
President of the Local Organising Committee
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Sunday 13th December 2009: Scientific Sessions and Social Programme
Monday 14th December 2009: Morning - Free Time and Social Programme
Afternoon - Scientific Sessions & Closing

**Contact Information:** Dr Charareh Pourzand ,Department of Pharmacy & Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY;
Phone: +44 1225 383 590; Fax: +44 386 114 ; Email: prscap@bath.ac.uk

# Green tea extract and epigallocatechin 3-gallate reduced labile iron pool and protected oxidative stress in iron-loaded HepG2 cells

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**Abstract**

Green tea (GT) contains polyphenolic catechins, which epigallocatechin 3-gallatte (EGCG) is the major active ingredients. Their free-radical scavenging and iron-chelating activities can protect human beings from many pathogeneses such as cancer, hypertension, diabetes and aging. β-thalassemia patients suffer from secondary iron overload caused by regular transfusions and increase of duodenal iron absorption. Iron is a chemical catalyst of Haber-Weiss and Fenton reactions that contributes reactive oxygen species (ROS) production and oxidative stress. In this study we purposed to investigate effect of GT crude extract and EGCG on oxidative stress and iron overload in cultured HepG2 cells and primary hepatocytes. Levels of labile iron pool (LIP) in the treated hepatocytes were measured with calcein fluorescence technique. Toxicity of the green tea product to the treated cells was determined using MTT assay. Apparently, the crude extract and EGCG reduced the LIP concentration in concentration-dependent manner and showed lower cytotoxicity than used standard iron chelators, desferrioxamine and deferiprone.

**Keywords:** Green tea, catechin, β-thalassemia, oxidative stress, iron

**5. ความเห็นและข้อเสนอแนะ**

ผู้วิจัยได้ดำเนินการตามแผนที่วางไว้จนได้ผลลัพธ์และผลผลิตเกิดขึ้นจากการศึกษาวิจัยครั้งนี้ แม้ว่าบางครั้งอาจต้องพบอุปสรรคและปัญหาสำคัญบางประการ แต่ก็ได้ปรึกษาหารือกับนักวิจัยพี่เลี้ยงจนสามารถดำเนินการวิจัยมาจนเสร็จสิ้น อย่างไรก็ตามผู้วิจัยมีความมุ่งมั่นและจุดประสงค์ที่จะทำการศึกษาวิจัยต่อยอดจากงานวิจัยนี้เพื่อให้เกิดประโยชน์สูงสุด ซึ่งสามารถนำไปใช้ประโยชน์และแก้ไขปัญหาด้านสุขภาพของผู้ป่วยธาลัสซีเมียต่อไป

ลงนาม

(รศ. ดร. สมเดช ศรีชัยรัตนกูล)

หัวหน้าโครงการวิจัยผู้รับทุน

## 6. แผนการดำเนินงานที่วางไว้

| กิจกรรม | ปี 2550 | | | | | | | ปี 2551 | | | | | | | | | | | ปี 2552 | | | | | | | | | | | ปี 2553 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | มิย | กค | สค | กย | ตค | พย | ธค | มค | กพ | มีค | เมย | พค | มิย | กค | สค | กย | ตค | พย | ธค | มค | กพ | มีค | เมย | พค | มิย | กค | สค | กย | ตค | พย | ธค | มค | กพ | มีค | เมย | พค |
| 1.เตรียมสารสกัดหยาบและ EGCG จากชาเขียว | / | / | / | | | | | | | | | | | | | / | | | | | | | | | | | | | | | | | | | | |
| 2.แยกกรดไขมันโอเมก้า3 | | | | / | / | / | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3.ศึกษาผลของชาเขียวช่วยยืดอายุเม็ดเลือดแดงของหนูธาลัสซีเมียที่ถูกเติมธาตุเหล็กเข้าไป | | | | | | | / | / | / | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 4.ศึกษาผลของสารสกัดหยาบและสารคะเตชินจากชาเขียวและกรดไขมันโอเมก้า3 ในหนู | | | | | | | / | / | / | / | / | / | / | / | / | / | | | | | | | | | | | | | | | | | | | | |
| 5.ศึกษาผลของสารสกัดหยาบและสารคะเตชินจากชาเขียว ลดปริมาณธาตุเหล็กรูป LIP และออกซิเดทีฟสเตรสของหนูธาลัสซีเมีย | | | | | | | | | | | | | / | / | / | / | / | / | | | | | | | | | | | | | | | | | | |
| 6.รวบรวมวิเคราะห์ข้อมูลที่ได้จากงานวิจัยเรื่องที่ | | | | | | | | | | | | | | | | | | | / | / | | | | | | | | | | | | | | | | |

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| 7.เขียนManuscript งานวิจัยเรื่องที่ 1 ตีพิมพ์ในวารสาร | | | | | | | | | | | | | | | | | | | | | | / | | | | | | | | | | | | | | | |
| 8.ศึกษาผลของสารสกัดหยาบและสารคะเตชินของชาเขียวในการลดระดับธาตุเหล็กรูป LIP และออกซิเดทีฟสเตรสในเซลล์ตับเพาะเลี้ยง | | | | | | | | | | | | | | | | | | | | | | | / | / | / | / | | | | | | | | | | | |
| 9.ศึกษาผลของสารสกัดหยาบและสารคะเตชินจากชาเขียวในการลดระดับธาตุเหล็กรูป LIP และออกซิเดทีฟสเตรสในเซลล์กล้ามเนื้อหัวใจเพาะเลี้ยง | | | | | | | | | | | | | | | | | | | | | | | | | | | / | / | / | / | | | | | | | |
| 10.รวบรวมและวิเคราะห์ข้อมูลทั้งหมดจากงานวิจัยเรื่องที่ 2 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | / | / | / | | | | |
| 11.เขียนรายงานฉบับสมบูรณ์ | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | / | / | | | |
| 12.เขียน Manuscript งานวิจัยเรื่องที่ 2 ตีพิมพ์ในวารสาร | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | / | / | / |

เอกสารแนบหมายเลข 4