Effect of Green Tea on Iron Status and Oxidative Stress in Iron-Loaded Rats

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Abstract: Plasma non-transferrin bound iron (NTBI) is potentially toxic and contributes to the generation of reactive oxygen species (ROS), consequently leading to tissue damage and organ dysfunction. Iron chelators and antioxidants are used for treatment of thalassemia patients. Green tea (GT) contains catechins derivatives that have many biological activities. The purpose of this study was to investigate the iron-chelating and free-radical scavenging capacities of green tea extract *in vivo*. Rats were injected ip with ferric citrate together with orally administered GT extract (GTE) for 4 months. Blood was collected monthly for measurement of iron overload and oxidative stress indicators. Plasma iron (PI) and total iron-binding capacity (TIBC) were quantified using bathophenanthroline method. Plasma NTBI was assayed with NTA chelation/HPLC. Plasma malonyldialdehyde (MDA) was determined by using the TBARS method. Erythrocyte oxidative stress was assessed using flow cytometry. Levels of PI, TIBC, NTBI and MDA, and erythrocyte ROS increased in the iron-loaded rats. Intervention with GT extract markedly decreased the PI and TIBC concentrations. It also lowered the transferrin saturation and effectively inhibited formation of NTBI. It also decreased the levels of erythrocyte ROS in week 4, 12 and 16. Therefore, green tea extract can decrease iron in plasma as well as eliminate lipid peroxidation in plasma, and destroy formation of erythrocyte ROS in the rats challenged with iron. The bifunctional effects could be beneficial in alleviating the iron and oxidative stress toxicity. In prospective, these GTE activities should be further examined in thalassemic animals or humans.

Key Words: Green tea extract, non-transferrin bound iron, free radical, iron, malonyldialdehyde.

INTRODUCTION

Iron overload is caused by multiple blood transfusion and excess gastrointestinal absorption, leading to most of the mortality and morbidity associated with thalassemia [1]. It can cause tissue damage and ultimately dysfunction of visceral organs (mainly in the heart, liver, and endocrine glands) [2]. A highly toxic form of iron, non–transferrin-bound iron (NTBI), is formed when the iron-binding capacity of transferrin has been exceeded. Uptake of the plasma NTBI into tissues contributes to increased intracellular labile iron pool (LIP) [3,4]. Potentially, it can catalyze the formation of reactive oxygen species (ROS) through the Fenton reaction. Patients with thalassemia have very high plasma levels of malonyldialdehyde (MDA), a by-product of lipid peroxidation. High levels of the MDA correlate positively with plasma iron (PI) and with NTBI [3]. NTBI can be quantified and used as a clinically useful test for monitoring iron-chelation therapy. Nowadays, desferoxamine (DFO) and deferiprone (DFP) are iron chelators used for treatment of thalassemia patients; however, their side effects are reported [5]. A novel oral chelator, deferasirox (ICL670), has been assessed in a comprehensive series of multi-center clinical trial [6,7]. Iron chelation therapy aims ultimately to decrease NTBI in plasma and LIP in the vital organs, particularly in the liver and heart.

Antioxidants may be an effective alternative therapy. These compounds have salutary effects on erythrocytes that have been damaged by oxidation [8]. Polyphenols (a major component of tea) binds to ferric ion and also protects thalassemic erythrocytes from oxidation [9-13]. They inhibit iron absorption, which may impair the utilization of dietary iron. They also have anti-oxidative properties due to chelation of pro-oxidant metals like iron. Catechins including epigallocatechin (EGC), catechin (C), epicatechin (EC), epigallocatechin 3-gallate (EGCG) and epicatechin 3-gallate (ECG) have been found in green tea (GT) infusions. The galloyl group of phenolic compounds has been implicated as the structure responsible for such inhibition [14]. Due to its high level, EGCG is widely accepted as the major anti-oxidant in green tea. Recent *in vitro* studies have shown that GT extract (GTE), as well as EGCG and ECG fractions can chelate plasma NTB and effectively decrease erythrocyte oxidative stress [10,15]. Contradictory, EGCG has a low inhibitory effect on non-heme iron absorption [16]. Importantly, the lipophilic permeable EGCG that is a main polyphenol constituent of green tea possesses anti-oxidative iron-chelating properties [14,17]. Due to oxidative stress and iron overload conditions in transfusion-dependent β-thalassemia major patients, combination of effective iron-chelating agent with

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anti-oxidant would be very helpful for the patients. In this study, we investigated the iron-chelating and free-radical scavenging capacities of GTE *in vivo*. For this purpose, we intraperitoneally (ip) loaded iron to the rats and monitored the levels of iron overload and oxidative stress parameters in their blood.

MATERIALS AND METHODS

Chemicals and Reagents

All catechins (EGC, C, EC, EGCG and ECG), aluminium chloride hexahydrate, 3-[N-morpholino]propanesulfonic acid (MOPS) and hydrogen peroxide were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). HPLC-grade acetronitrile and methanol were purchased from Merck Company. All other chemicals and reagents used were of AnalaR grade. CP22 (1-methyl-2-propyl-3-hydroxy-pyridin-4-one) was kindly donated by Dr. Chada Phisalaphong, Thailand Government Pharmaceutical Organization, Bangkok. A stock of ferric nitrate (1 ppm or 18.16 mM iron in 1% nitric acid) was used as the iron source for other preparations. Stock ferric citrate and ferric nitrilotriacetate solutions were prepared by consecutive mixing of ferric nitrate with the chelators, citric acid and nitrilotriacetic acid (NTA), (at a 1:5 molar ratio of Fe^{3+} to chelator). Various iron concentrations were prepared in 10 mM MOPS buffer, pH 7.0, just before use.

Preparation of Green Tea Extract

Fresh tea shoots were harvested, immediately dried in a microwave oven and quantified for catechins content according to the method recently reported by Srichairatanakool and colleagues [10]. The green tea (GT) catechin derivatives were analyzed using an HPLC system consisting of a ternary delivery dual-pump (Model 3500; LDC Milton-Roy Analytical Inc., Rochester, NY, USA) equipped with a flow cell detector (SpecMonitor 3200, LDC Milton-Roy Analytical Inc.). The GT (2.0 g in dry weight) were extracted in 100 ml of hot water (80°C) for 10 minutes. The extract was then passed through a filter membrane (cellulose acetate type, 0.45 μm pore size; Whatman International Ltd, Maidstone, England). The extract (2.0 g%, w/v) (50 μl) was fractionated on the analytical column (Spherisorb ODS2, 250 x 4.7 mm, 5 μm) capped with a guard column (Spherisorb ODS2, 10x4.7 mm, 5 μm). Individual catechins were eluted isocratically with a mobile phase solvent (0.05% H_2SO_4:acetonitrile:ethyl acetate = 86:12:2, v/v/v) at a flow rate of 1.0 ml/min. and monitored at 280 nm. Authentic EGC, EC, C, EGCG, and ECG standards at the concentrations of 0-10 mM were used to calibrate the column and to build standard curves. The identification and determination of each catechin concentration (mg/g dry weight) was performed by comparing their retention times with those of the standards. The GT extract containing EGC (13.4 mg/g dry weight), EC (9.4 mg/g), C (38.9 mg/g), EGCG (58.7 mg/g) and ECG (7.1 mg/g) (12% EGC, 8% EC, 34% C, 50% EGCG and 6% ECG) was given to the animals throughout this study.

Animal Protocol

The study has been approved by the Animal Ethical Committee of the Medical Faculty, Chiang Mai University (Reference Number 03/2548). The protocol of iron loading was published previously [18]. Male Wistar rats (initial weight 60-70 grams) were purchased from National Laboratory Animal Center, Mahidol University, Salaya Campus, Nakornpathom. After acclimatization, the rats were housed in stainless steel cages at ambient temperature (20-22°C), humidity (50±10%) and controlled lighting (12-hour day/night cycle), then divided into four groups (12 animals each). Group I (control) was untreated, Group II was intraperitoneally injected with ferric citrate, pH 7.0 solution (5 mg/kg body weight/week), Group III was injected intraperitoneally with the ferric citrate solution (5 mg/kg/week) and administered orally with GT crude extract (300 mg/kg/week) and Group IV was injected intraperitoneally with the ferric citrate solution (5 mg/kg/week) and administered orally with DFP (15 mg/kg/week). The rats were fed with normal diet and deionized water *ad libitum* for 16 weeks.

Blood samples were collected from tail vein aseptically at indicated time. At the end of the study, the rats were anesthetized under diethyl ether atmosphere and sacrificed by cardiac puncture to collect the blood into lithium heparin-coated tubes. The blood was centrifuged at 3,000rpm, 25°C for 15 minutes. Plasma was removed and kept frozen at -20°C for further analysis.

Measurement of Plasma Iron

Plasma iron (PI) concentration was measured using the bathophenanthroline method developed by the International Committee for Standardization of Haematology (ICSH) [19]. Plasma and standard iron solution (0.5 ml) were incubated with 10% trichloroacetic acid (TCA) solution (33.3 ml of thioglycolic acid, 98.4 ml of TCA, 2 mol of HCl and 700 ml of deionizaed water) (0.5 ml) at room temperature for 5 minutes. The mixture was subsequently centrifuged at 6,000 rpm, 4°C for 10 minutes to achieve a clear supernatant. The supernatant (0.5 ml) was mixed with a chromogen solution (250 mg of disodium bathophenanthroline disulfonic acid and 84.04 g of sodium acetate in 1.0 liter of deionized water) (0.5 ml), and allowed to stand at room temperature for 5 minutes for color development. The absorbance was read against reagent blank at 540 nm using a UV-VIS Double-beam spectrophotometer (Shimadzu Corporation, Analytical & Measuring Instrument Division, Kyoto Japan). Stock iron solution (5 mM ammonium ferrous sulfate in 10 mM H_2SO_4) was initially prepared, then diluted serially with acetate buffer, pH 5.0 solution to reach concentrations of 0-100 μM.

Determination of Total Iron-Binding Capacity

Total iron-binding capacity (TIBC) was determined using the method developed by the ICSH 1978 [20]. In the assay, ammonium ferric citrate solution (40 mg/dl) (10 μl) was added to the plasma (0.5 ml) and incubated at room temperature for 30 minutes. Light magnesium carbonate powder (0.4-0.5 g) was added, mixed on a rotary mixer for at least 15 minutes and centrifuged at 6,000 rpm, 4°C for 10 minutes. Supernatant (0.2 ml) was treated with the TCA solution (0.2 ml) for precipitation of protein, and the mixture was centrifuged at 6,000 rpm, 4°C for 10 minutes. The supernatant was incubated with the bathophenanthroline solution, the absorbance was measured as described above. In addition, trans-

ferrin saturation (TS) was calculated by dividing the PI by the TIBC.

NTA CHELATION/HPLC-BASED MEASUREMENT OF NON-TRANSFERRIN BOUND IRON

Plasma NTBI measurement was undertaken essentially as described by Singh and colleagues [21]. Briefly, plasma (450 µl) was incubated with 800 mM NTA, pH 7.0 solution (50 µl) at room temperature for 30 minutes to transform NTBI into Fe-NTA. Afterwards, plasma proteins were removed by centrifugation of the treated plasma using an ultracentrifugation filtration device (NanoSep®, 30-kDa cut off, polysulfone type; Pall Life Sciences, Ann Arbor, MI USA) at 12,000 rpm (10620*g*, Hettich Centrifugation, Germany), 15°C for 45 minutes. The ultrafiltrate was analyzed using a non-metallic HPLC system. The HPLC conditions were as follows: a dual-piston high pressure pump (ConstaMetric®3500 LDC Analytical, Inc., Florida, USA), a glass analytical column (ChromSep ODS1, 100x10 mm, 5 µm; Chrompack International, Middelburg, the Netherlands), mobile-phase solvent containing 3 mM CP22 (1-methyl-2-propyl-3-hydroxypyridin-4-one) in 19% acetonitrile (HPLC grade, E. Merck, Darmstadt, Germany) buffered with 5 mM MOPS, pH 7.0 at a flow rate of 1.0 ml/min. Column effluents were monitored at 450 nm using a flow-cell detector (SpecMonitor2300; LDC Milton-Roy Inc., Florida, USA) and conducted with the BDS software (BarSpec Ltd., Rehovot, Israel). The NTBI peak was calculated from a calibration curve of standard iron solutions (0-16 µM Fe-NTA in 80 mM NTA, pH 7.0). The NTA solution (800 mM) was prepared by adding N,N-bis[carboxymethyl]glycine disodium (purity minimum 99%, Sigma-Aldrich Co., St. Louis, MO, USA) solution (800 mM) to N,N-bis[carboxymethyl]glycine trisodium (purity 98%, Sigma-Aldrich Chemie GmbH, Steinheim, Switzerland) solution (800 mM) to reach a final pH of 7.0.

The NTBI measurement was also slightly modified by including an aluminum blocking step to saturate free iron-binding sites on iron chelator molecule and preventing iron shuttling by NTA when excess unbound iron chelator remained in chelator-treated plasma [22]. Aluminium chloride solution (200 µM) was added to chelator-treated plasma, and the mixture was further incubated for 1 hour. The plasma was then quantified for NTBI as described above.

Thiobarbituric Acid Reactive Substance Assay

One lipid peroxidation product, malonyldialdehyde (MDA) was measured using the thiobarbituric acid reactive substances (TBARS) assay [23]. Plasma or standard solution (375 µl) was mixed with 0.2% butylated hydroxytoluene (BHT) solution (40 µl). The solution was split into two aliquots (125 µl each) and 750 µl of phosphoric acid (0.44 M) was added. Deionized water (750 µl) was added to the reagent blank tube, whereas thiobarbituric acid solution (1% in 0.05 M NaOH) (750 µl) was added to the tube containing the unknown. All tubes were incubated at 100°C for 30 minutes, then cooled down to room temperature. Absorption was measured at 560 nm against a reagent blank. The amount of TBARS was calculated as MDA equivalents using 1,1,3,3-tetramethoxypropane as the reference standard.

Measurement of Erythrocyte Oxidative Stress

Erythrocyte oxidative stress was assayed basically as described by Amer and colleagues [24]. Briefly, RBC suspension (40% hematocrit) was prepared in the PBS solution. The red cell suspension (2 µl) was diluted with 9 ml of PBS. The RBC suspension (2 µl) was incubated with a 2',7'-dichlorofluorescein diacetate (DCFH-DA) solution (10 mg/ml in methanol) at 37°C under 5% CO_2 atmosphere for 15 minutes. The RBC was then washed three times with the PBS solution and subsequently exposed to 3% H_2O_2 for 15 minutes before being analyzed for green fluorescence on a FACscan Becton Dickinson (Mountain View, CA, U.S.A.) flow cytometer. Geometric mean fluorescence channel (MFC) was derived by CellQuest® software. Increased green fluorescence intensity (FI) indicates increased intracellular oxidative stress.

Statistical Analysis

The data were expressed as mean±standard deviation. The statistical difference of analyzed data was determined by using the Student's *t*-test.

RESULTS AND DISCUSSION

Plasma Iron

As shown in Fig. **1**, the levels of PI increased in the rats loaded with iron in time-dependent manner when compared to the unloaded rats. The PI levels were considerably lower in the GT-treated rats than the untreated rats (51.5±1.2 µM and 55.5±8.9 µM in week 4, 45.7±1.2 µM and 69.5±2.3 µM in week 8, 70.0±7.0 µM and 110.9±9.2 µM in week 12, and 76.6±2.3 µM and 115.5±5.1 µM in week 16. DFP was able to decrease the PI levels but less effectively than the GTE. This indicates that iron administration increases the PI levels whereas the GTE and DFP can abolish iron loading in the rats.

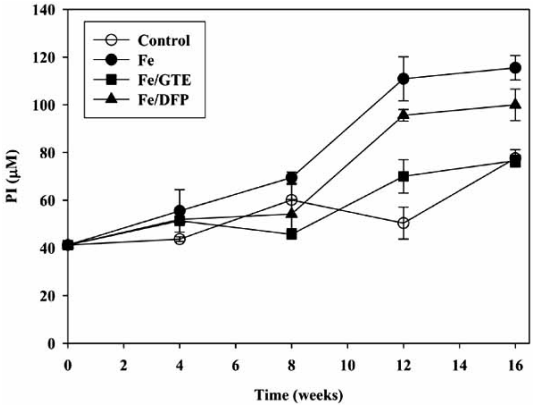

Fig. (1). Levels of PI in rats (n = 12/group) untreated (open circle) or treated with iron alone (closed circle), iron and GTE (closed square), iron and DFP (closed triangle). Data are expressed as mean±SD.

Plasma Total Iron Binding Capacity and Transferrin Saturation

Normally, the plasma TIBC value is decreased when PI concentration is increased. It was found that the iron treat-

ment lowered the plasma TIBC, and the GT extract abolished lowering of the TIBC during iron loading conditions. The DFP also increased the plasma TIBC, particularly in week 12 and 16 (Fig. **2**). When the rats were treated with the iron, their plasma transferrin saturation values gradually increased and reached full saturation in week 16. Interestingly, plasma transferrin saturation was approximately one-half lower in the GT-treated rats than in the rats without GT treatment. Similarly, DFP lowered the plasma transferrin saturation, but less effectively than the GT extract (Fig. **3**). These results suggest that the GT extract could act as an iron chelator like DFP and interfere the iron loading onto plasma transferrin.

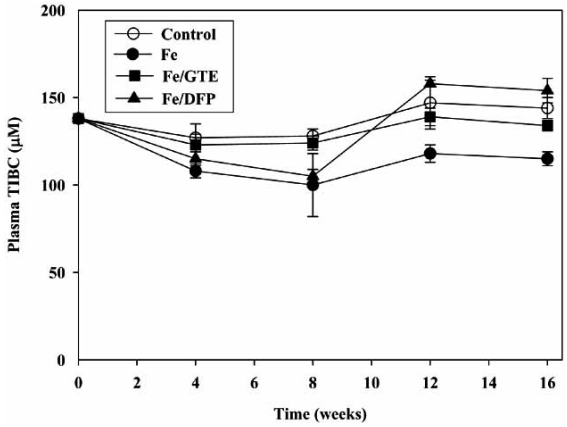

Fig. (2). Levels of plasma TIBC in rats (n = 12/group) untreated (open circle) or treated with iron alone (closed circle), iron and GTE (closed square), iron and DFP (closed triangle). Data are expressed as mean±SD.

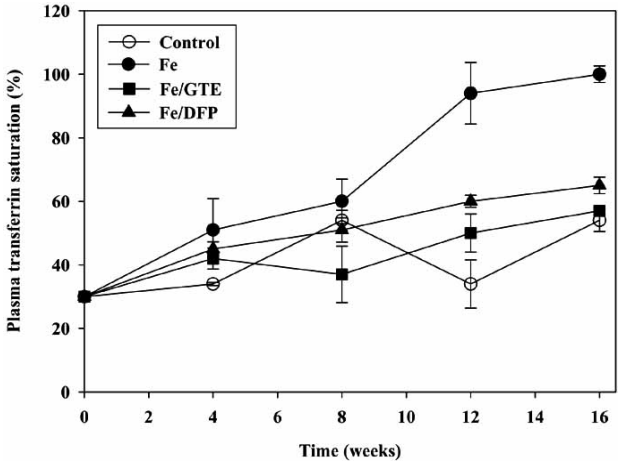

Fig. (3). Levels of plasma transferrin saturation in rats (n = 12/group) untreated (open circle) or treated with iron alone (closed circle), iron and GTE (closed square), iron and DFP (closed triangle). Data are expressed as mean±SD.

Plasma Non-Transferrin Bound Iron

In Fig. **4** when transferrin in the plasma is unsaturated, NTBI normally is undetectable (<0 μM) (open circle). Iron loading can occupy free iron-binding sites onto the transferrin molecule and consequently lead to saturation. Hitherto,

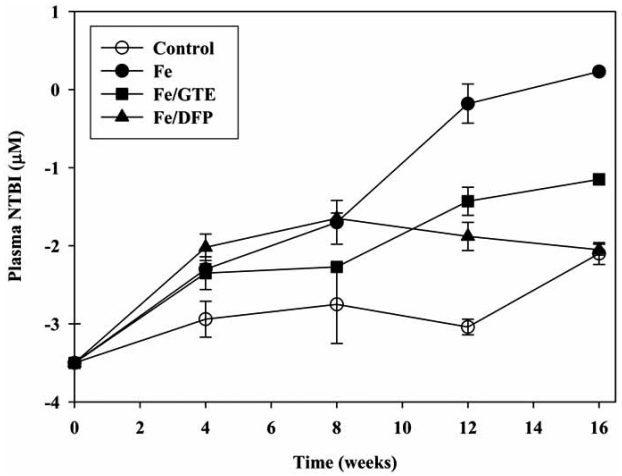

Fig. (4). Levels of plasma NTBI in rats (n = 12/group) untreated (open circle) or treated with iron alone (closed circle), iron and GTE (closed square), iron and DFP (closed triangle). Data are expressed as mean±SD.

the NTBI was measurable at (0.23±0.04 μM) in the rats receiving iron up to 16 weeks (closed circle). On the other hand, NTBI was neither detectable in the plasma of the rats treated with GT extract (closed square) nor with DFP (closed triangle). Clearly, the GT extract decreased the concentrations of plasma NTBI. The longer DFP was administered to the iron-loaded rats, the more effective it inhibited the generation of plasma NTBI.

Plasma Thiobarbituric Acid Reactive Substances

Without iron treatment levels of plasma MDA tended to increase slightly, probably due to nutritional or environmental factors. When the iron was administered, levels of the plasma MDA were about two-fold increased from 4.0±0.1 μM (at the beginning) to 8.6±1.5 μM (week 4), giving higher amount of plasma MDA in week 4, 12 and 16. However, the GT extract administration and DFP abolished such plasma lipid peroxidation (Fig. **5**). These results lead us to postulate that the GTE not only binds the iron but also scavenges

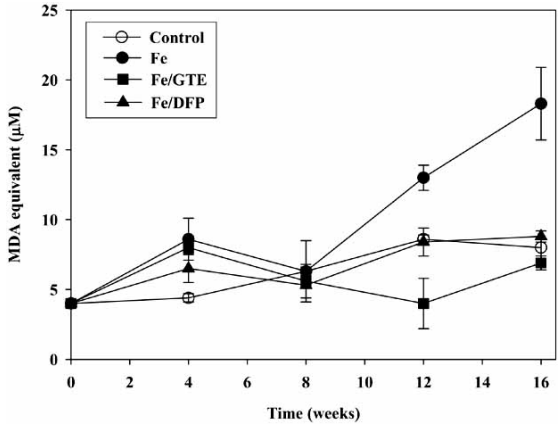

Fig. (5). Levels of plasma TBARS (MDA equivalent) in rats (n = 12/group) treated (open circle) or treated with iron alone (closed circle), iron and GTE (closed square), iron and DFP (closed triangle). Data are expressed as mean±SD.

the produced lipid peroxidation product represented as the MDA.

Erythrocyte Oxidative Stress

We started measuring amount of the ROS in rat erythrocytes in week 3 and followed until week 16. Iron treatment enhanced generation of the erythrocyte ROS in Group 2 rats in week 8 (125±15%), week 12 (202±22%) and week 16 (196±29%). However, green tea effectively decreased the amount of erythrocyte ROS (90±5%, 171±21% and 90±5% respectively) in Group 3 rats in such time periods. Though DFP (15 mg/kg) was unable to lower the erythrocyte ROS (188±18%) in week 4, it scavenged free radicals (145±19%) more efficiently in week 12 and (51±17%) in week 16 than the GTE (Fig. **6**).

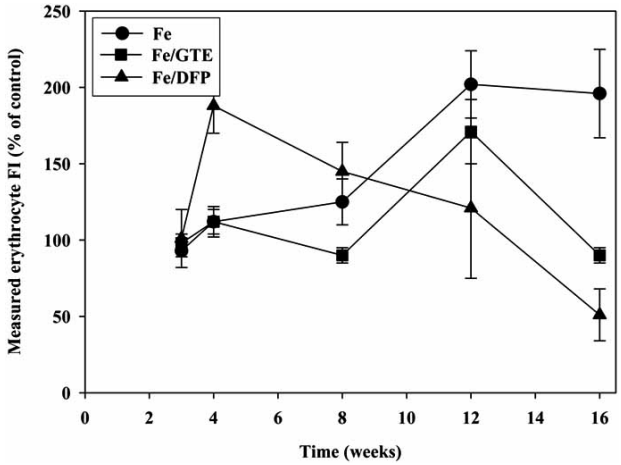

Fig. (6). Levels of erythrocyte oxidative stress in rats (n = 12/group) treated with iron alone (closed circle), iron and GTE (closed square), iron and DFP (closed triangle). Data are expressed as mean±SD of FI compared with the control rats.

Based on the results of this study it can be concluded that green tea extract may reduce plasma NTBI levels in conditions of thalassemia and inhibit iron-induced generation of ROS *in vivo*, consistent with previous *in vitro* studies [10,25]. Flavonol or catechin polyphenols are more commonly found in tea and comprise 30-40 percent of the extractable solids of dried green tea. They require catechol moiety on ring B and 3-OH moiety in combination with C2 C3 double bond for free radical scavenging and iron chelating, respectively [26]. Other anti-oxidant procyanidin, caffeine and theophylline are also found in green tea; however, there were not any evidence to support their iron-chelating capability. A recent study supports beneficial anti-oxidative property of EGCG in attenuating oxidative damages in aged rats [27]. Our investigated GTE had a high content of the EGCG (50%) and a current study has demonstrated the iron-chelating capacity of the EGCG [28]. EGCG is a major active ingredient and the most abundant in GTE and ECG is the second most abundant. A previous study shows that hydroxyl-radical scavenging capabilities of the catechin species were ECG > EC > EGCG >> EGC, and a ratio of the EGC, EGCG, ECG or EC to Fe^{3+} was 3:2, 2:1, 2:1 and 3:1 respectively [29,30]. Both of them have galloyl group(s) in the molecules and have strong anti-oxidative and iron-chelating capacities [15,31]. Their plasma concentrations before administration of the GT were less than 2 nM. After ingestion of the GT for 90 minutes, the plasma EGCG and ECG concentrations dose-dependently increased, reached the highest level about 120 minutes, and then decreased [32,33]. Increase in plasma catechin level was functionally significant for dose-response rise in anti-oxidant capacity and free-radical scavenging ability in the plasma compartment [34].

In this study, iron was gradually accumulated in the untreated rats while it was counteracted by the administration of GTE and DFP. It is possible that these two compounds interfere the intestinal iron absorption and remove the plasma iron; particularly, the EGCG and ECG galloyl group(s) could play an important role in binding the iron. The plasma NTBI was correlated positively with the plasma MDA and erythrocyte ROS. Levels of the plasma NTBI, plasma MDA and erythrocytes ROS responded in a dose-dependent manner upon treatment with GTE and DFP. Consistently, iron overload in thalassemia patients produces marked amounts of serum MDA [35]. The galloyl group(s) would attack the plasma NTBI and destroy ROS produced in many diseases. However, previous studies have shown that regular ingestion of tea did not inhibit lipid peroxidation in humans, whereas GTE and EGCG were pro-oxidant [36,37].

The effects of GT catechins on iron status and oxidative indices appear very promising in animal models, but the data in animals and humans with thalassemia are limited. Further studies examining the effects of GTE and catechin intake on biomarkers of oxidative damage to lipids, proteins, and DNA are needed. This approach, if successful, could be particularly useful for chelation therapy in countries with limited financial resources.

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