Efficacy of Curcuminoids in Alleviation of Iron Overload and Lipid Peroxidation in Thalassemic Mice

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Abstract: Non-transferrin bound iron (NTBI) is detectable in plasma of β-thalassemia patients and participates in free-radical formation and oxidative tissue damage. Desferrioxamine (DFO), deferiprone (DFP) and deferasirox (DFX) are iron chelators used for treatment of iron overload; however they may cause adverse effects. Curcuminoids (CUR) exhibits many pharmacological activities and presents β-diketone group to bind metal ions. Iron-chelating capacity of CUR was investigated in thalassemic mice. The mice (C57BL/6 stain); wild type ($^{mu}\beta^{+/+}$) and heterozygous β-knockout ($^{mu}\beta^{th-3/+}$) were fed with ferrocene-supplemented diet for 2 months, and coincidently intervened with CUR (200 mg/kg/day) and DFP (50 mg/kg/day). Plasma NTBI was quantified using NTA chelation/HPLC method, and MDA concentration was analyzed by TBARS-based HPLC. Hepatic iron content (HIC) and total glutathione concentration were measured colorimetrically. Tissue iron accumulation was determined by Perl's staining. Ferrocene-supplemented diet induced occurrence of NTBI in plasma of thalassemic mice as well as markedly increased iron deposition in spleen and liver. Treatment with CUR and DFP decreased levels of the NTBI and MDA effectively. Hepatic MDA and nonheme iron content was also decreased in liver of the treated mice whilst total glutathione levels were increased. Importantly, the CUR and DFP reduced liver weight index and iron accumulation. Clearly, CUR is effective in chelation of plasma NTBI in iron-loaded thalassemic mice. Consequently, it can alleviate iron toxicity and harmfulness of free radicals. In prospective, efficacy of curcumin in removal of labile iron pool (LIP) in hepatocytes and cardiomyocytes are essential for investigation.

Key Words: Curcuminoids, non-transferrin bound iron, β-thalassemia, iron overload, oxidative stress.

INTRODUCTION

Transfusion-dependent β-thalassemia patients suffer from secondary iron overload and oxidative stress [1]. The capacity of transferrin to bind iron is often exceeded, leading to formation of non-transferrin-bound iron (NTBI) species. NTBI has the potential to generate reactive oxygen species (ROS) through the Haber-Weiss and Fenton reactions [2]. NTBI also delivers iron to certain, tissues leading to excess iron accumulation. Human tissues contain superoxide dismutases, catalase, and glutathione peroxidase that can counteract the free radicals-induced oxidative damage; nonetheless, the anti-oxidative defense mechanisms may not be sufficient to prevent damage in β-thalassemia patients with large iron burdens [3, 4]. Thus high plasma levels of malondialdehyde (MDA), a product of lipid peroxidation, are present in thalassemic patients [5]. Without effective chelation therapy the highly accumulated iron results in progressive dysfunction of the heart, liver and endocrine glands [6, 7]. Iron-induced liver disease is another cause of death, particularly in older patients [8]. Within two years of blood transfusions, increased hepatic collagen formation and liver fibrosis have been observed [9, 10], cirrhosis has been observed as early as the first decade of life [11-13].

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Though desferrioxamine (DFO) and deferiprone (DFP) are useful iron chelators for treatment of iron overload, they may exert adverse effects and are not effective in all patients. Iron chelating agents may act by removing the iron pools in the plasma compartment, such as iron released from macrophages after the catabolism of red cells or directly on NTBI species. Intracellular iron pools, particularly the labile iron pool (LIP) are important targets, the liver containing the majority of storage iron in transfusional iron overload. Both DFO and DFP have short plasma half lives, so that NTBI and labile intracellular pools are only accessed intermittently when these agents are given as monotherapy. NTBI rebounds rapidly after DFO or DFP acre cleared from the plasma, leading to rebound high levels of labile iron. The addition of other chelating agents may decrease exposure to labile iron species, thereby decreasing tissue damage. If such agents are conveniently and safely administered this would have additional advantages.

Curcuminoids (CUR) are present tumeric of *Curcuma longa* Linn. Commonly used as a spice in cooking. CUR extracts, of which curcumin is the major component, have been shown to possess antioxidant [14, 15], anti-inflammatory [14] and anticancer properties [16]. Genotoxic or teratogenic or other toxicities has not been absent in preliminary preclinical and clinical studies [17]. CUR and its derivatives are also capable of scavenging free radicals, chelat-

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ing and disarming oxidative metal ions [18]. Biological activities of the CUR have been attributed to hydroxyl group substituents on the benzene rings and to the diketonic structure, which can undergo a keto-enol tautomerisation [19]. Curcumin also binds iron to produce a Fe^{3+}-curcumin complex with a formation constant of 10^{22} [20]. A strong chelating ability towards many metal ions is thought to be related to the diketone stuucrure. These properties could allow CUR to be of great importance in treatment of iron overload and alleviation of oxidative stress. Interestingly, curcumin has been shown to bind Fe(III) in solution and also to chelate NTBI species in β-thalassemic serum [21]. It is hypothesized that curcuminoids use the diketone group to chelate iron that is distributed in plasma compartment as well as in iron overloaded tissues. In this study, we investigated iron-chelating and anti-oxidative activities of the curcuminoids in iron-loaded β-thalassemic mice.

MATERIALS AND METHODS

Chemicals and Reagents

Curcuminoids (83% curcumin content), DFP, CP22 (1-methyl-2-propyl-3-hydroxypyridin-4-one) and tragacanth were kindly donated by Dr. Chada Phisalaphong at the Government Pharmaceutical Organization, Bangkok, Thailand. 3-[N-morpholino]propanesulfonic acid (MOPS), thiobarbituric acid (TBA) and butylated hydroxytoluene (BHT) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). HPLC-grade acetonitrile and methanol were purchased from Merck Company. Other chemicals and reagents used were AnalaR grade. A stock of ferric nitrate (1 ppm or 17.86 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 mixing of ferric nitrate with citric acid and nitrilotriacetic acid (NTA) respectively at a molar ratio of Fe^{3+} to chelator at 1:5. Various iron concentrations were freshly prepared in 10 mM MOPS buffer, pH 7.0.

Animal Treatment

The study was approved by the Animal Ethical Committee of Medical Faculty, Chiang Mai University, Thailand (Reference Number -3/2548). Two strains of C57BL/6 mouse (60 days of age), wild type ($^{mu}\beta^{+/+}$) (WT) and heterozygous β-knockout ($^{mu}\beta^{th-3/+}$) (BKO), were obtained from Thalassemia Research Center, Institute of Science and Technology for Research and Development, Mahidol University, Salaya Campus, Thailand. After acclimatization, mice were housed in stainless steel cages at ambient temperature (20-22°C), humidity (50±10%) and controlled day/night cycle (12 hour/12 hour). The mice had free access to drinking water. For iron loading, WT and BKO mice were fed with ferrocene-supplemented chow diet (0.2%, *w/w*) (FE diet) for 60 days, then randomly divided into 3 groups (12 mice each). Group WT1 and Group BKO1 were orally administered the FE diet together with deionized water (placebo), Group WT2 and Group BKO2 were orally administered the FE diet together with curcuminoids (200 mg/kg/day), and Group WT3 and Group BKO3 were orally administered the FE diet together with DFP (50 mg/kg/day) for 60 days. Colloidal curcuminoid (0.5 %, *w/v*) was made from mixing up weighed curcuminoids in a targacanth solution previously prepared in deionized water. Heparinized blood was collected from tail vein every 20 days and centrifuged at 3000 rpm, 4°C for 15 minutes. Plasma was removed and kept frozen at -20°C for further analysis. At the end of the study the mice were sacrificed under ether anesthesia and their heart blood samples were collected. Their liver, heart and spleen were dissected, weighed and cut into small pieces, which one part was fixed in 10% formalin for histochemical examination and the other one was frozen at -80°C for further analysis.

Measurement of Blood Hemoglobin

Hemoglobin concentrations were determined using the cyanmethemoglobin method. Briefly, 2.5 µl of fresh blood was incubated with 625 µl of Drabkin's solution at room temperature for 15 minutes. Optical density of the colored product was measured at 540 nm against the Drabkin's solution as a reagent blank.

Determination of Plasma Non-Transferrin Bound Iron

Plasma NTBI concentrations were measured according to the NTA chelating/HPLC method [22] with slight modification. Plasma was incubated with NTA solution (a final concentration of 80 mM) pH 7.0 for 30 minutes at room temperature to produce a Fe^{3+}-$(NTA)_2$ complex. Afterwards, the Fe^{3+}-$(NTA)_2$ complex was separated from plasma proteins by spinning the plasma through a membrane filter (NanoSep®, 30-kDa cut off, polysulfone type; Pall Life Sciences, Ann Arbor, MI USA). The concentration of the Fe^{3+}-$(NTA)_2$ representing NTBI in the ultrafitrate was determined using a non-metallic HPLC system. Analytes were fractionated onto a glass analytical column (ChromSep-ODS1, 100×3.0 mm, 5 µm), eluted with mobile-phase solvent (3 mM CP22 in 19% acetronitrile/MOPS buffer pH 7.0) at a flow rate of 1.0 ml/min. The effluents were monitored at 450 nm using flow-cell detector (SpecMonitor2300; LDC Milton-Roy Inc., Florida, USA) and conducted with BDS software (BarSpec Ltd., Rehovot, Isael) The NTBI concentration was calculated from a calibration curve of standard iron solution (0-16 µM Fe-NTA in 80 mM NTA, pH 7.0).

Measurement of Hepatic Iron Content

Hepatic iron content (HIC) was determined using a colorimetric technique [23] and expressed as milligram of non-heme iron per gram of liver dry weight. Liver tissue was dried at 120°C for 24 hours and weighed. The liver tissue was digested with a mixture of concentrated sulfuric acid and concentrated nitric acid (1:1, *v/v*), and a final volume was adjusted to 10 ml with deionized water. The tissue iron was reduced with ascorbic acid and reacted with 2,4,6-tripyridyl-*s*-triazine (TPTZ) to form an intense violet-colored product. Optical density of the colored product was measured spectrophotometrically at 593 nm against reagent blank.

Thiobarbituric Acid Reactive Substance (TBARS) Assay

Concentrations of liver and plasma TBARS were determined by HPLC method as mentioned below [24]. 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 ice bath. A 0.5-ml aliquot of the homogenate was transferred to 1.1 ml of 10% (*w/v*) trichlo-

roacetic acid containing BHT (50 ppm), mixed well and heated at 90°C for 30 minutes. After cooling to room temperature, the mixture was centrifuged at low speed to achieve clear supernatant. The supernatant (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 to develop pink-colored product. For plasma, the sample (50 µl) was mixed with 2% BHT solution (2.5 µl), followed by 0.44 M H_3PO_4 (750 ml) and TBA solution (0.6% *w/v*) (250 µl), incubated at 90°C for 30 minutes and allowed to cool down. The pink-colored solution was filtered through 0.45-µm syringe filter and analyzed with the HPLC system using analytical column (Water Spherosorb ODS2, 250x4.3 mm, 5 µm), mobile-phase solvent of 50 mM KH_2PO_4: methanol (65:35, *v/v*) at a flow rate of 1.0 ml/min. Eluents were detected on-line at 532 nm. A standard curve was constructed from the peak height (y-axis) of various concentrations of standard 1,1,3,3-tetramethoxypropane (x-axis). Liver and plasma TBARS concentrations were determined from the standard curve and reported as MDA equivalents.

Liver Glutathione Concentration

Liver tissue was deproteinized with 5-sulfosalicylic acid solution (5%). Clear supernatant was measured for glutathione concentration using a colorimetric method. Basically, reduced glutathione (GSH) converts 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) to 5-thio-2-nitrobenzoic acid (TNB), a yellow-colored product. Simultaneously, produced oxidized glutathione (GSSG) was reduced by glutathione reductase in the presence of NADPH to the GSH. Absorbance of the TNB was measured at 412 nm against reagent blank.

Histochemical staining for iron deposition in tissue

Iron deposition in liver, heart and spleen were analyzed by fixing the dissected organs in 10 % neutral-buffered formalin, embedding the tissue sections in paraffin boxes and cutting with a sliding microtome. The 5-µm thick tissue section was stained with acid potassium ferrocyanide solution (called Perl's staining solution) and examined under a simple microscope by an expert pathologist.

Statistical Analysis

The data are expressed as mean±standard deviation (mean±SD). Statistical difference of analyzed data was determined using Student's paired *t*-test using SPSS version 16.0 which $p < 0.05$ indicates statistical significance.

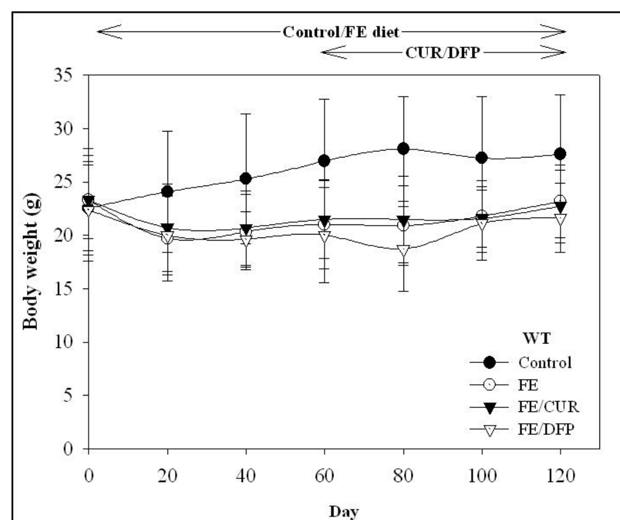
RESULTS AND DISCUSSION

Body Weights

In Fig. (**1**), body weights of the control diet-fed mice gradually increased for 60 days. In comparison, body weights of the mice administered with the high FE diet were significantly lower in both WT ($p < 0.001$) and BKO ($p < 0.05$) strains. Intervention with CUR or DFP did not show a significant effect on body weight in either strain of iron loaded mice however.

Hemoglobin Level

Hemoglobin levels in thalassemic mice were lower than wild type mice. As shown in Fig. (**2**), the iron- supplemented diet slightly increased hemoglobin level in both strains. Treatment with CUR and DFP did not alter the hemoglobin level in iron supplemented WT mice, whereas they slightly increased the level in iron overloaded BKO mice.



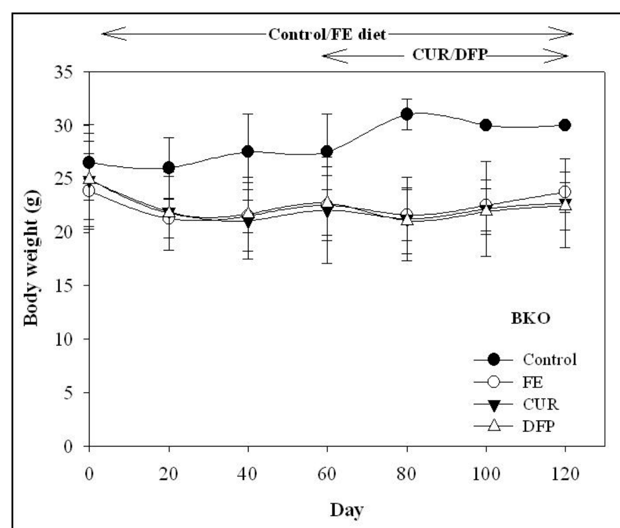
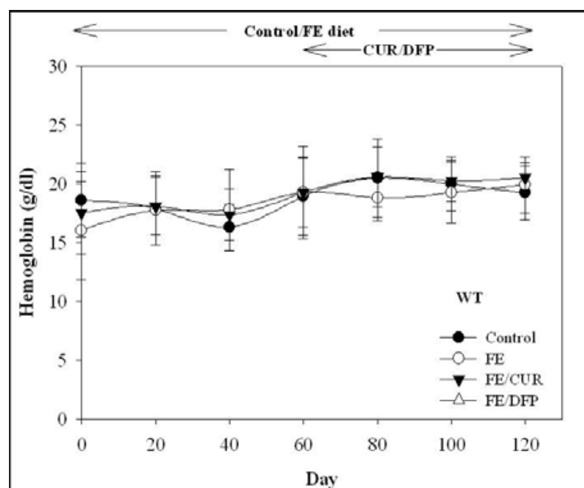


Fig. (1). Body weight of the WT mice (top) and BKO mice (bottom) fed with control and FE diets, together with CUR and DFP treatments. Data are expressed as mean±SD (n = 12).

Plasma Non-Transferrin Bound Iron

One potential action of an iron chelating agent is the lowering of plasma NTBI. In this study, plasma NTBI concentrations were measured in each group of mice, every 20 days. There were undetectable of NTBI levels in both WT and BKO mice at day 0. Unlike β-thalassemia major patients, where NTBI is present in a range of 1-12 µM, the BKO thalassemic mice used in this study did not have NTBI in plasma unless a high iron diet was administered. As demonstrated in Fig. (**3**), the mice fed with control diet showed no NTBI (<0 µM). By contrast, administration of a ferrocene-supplemented diet for 20 days significantly increased NTBI



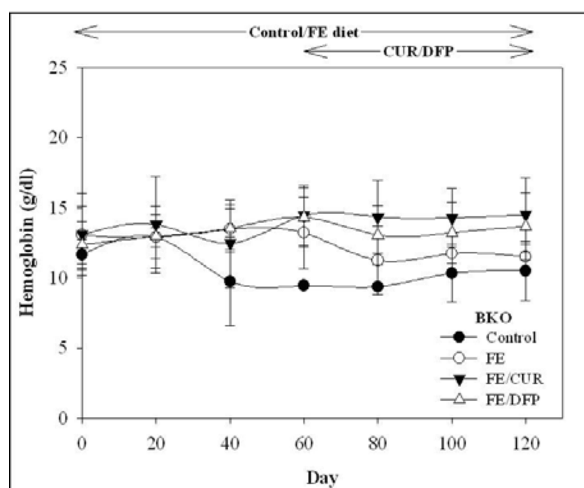
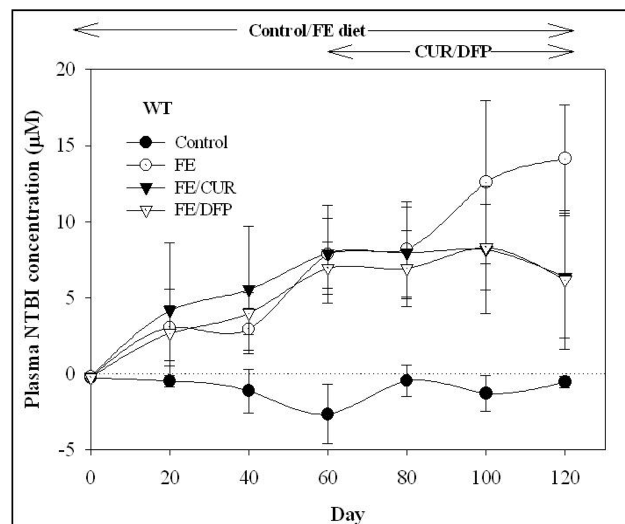


Fig. (2). Blood hemoglobin concentrations of the WT mice (top) and BKO mice (bottom) fed with control and FE diets, together with CUR and DFP treatments. Data are expressed as mean±SD (n = 12).

levels in WT (p <0.02) and in BKO mice (p <0.01). The NTBI concentration gradually increased in a time dependent manner. Moreover, it was found that NTBI levels in BKO mice fed with the ferrocene-diet were slightly higher than that in WT mice at all time intervals. These higher NTBI levels in BKO mice might be expected because of enhanced gastrointestinal absorption of iron as well as increased red blood cell destruction, similar to that seen in β thalassemia patients. In the following 60 days, intervention with CUR significantly suppressed NTBI levels, particularly in day 100 and 120. DFP also lowered plasma NTBI in both WT and BKO mice. These results suggest that CUR could act to decrease NTBI by the chelation of iron, as effectively as DFP.

Hepatic Iron Concentration (HIC)

Liver is the major organ for iron accumulation. One of characteristic manifestations of systemic iron overload is liver disease with eventual development of cirrhosis and hepatocellular carcinoma due to production of iron catalyzed free radicals. Hence, at the end of the experiment, we investigated whether CUR had decreased hepatic iron concentrations. As illustrated in Fig. (**4**), at baseline, before the iron treatment, nonheme iron content in liver of BKO mice was significantly higher than WT mice (p <0.001). This is presumably the consequence of increased iron absorption associated with ineffective erythropoiesis in the BKO mice. After day 60 of iron loading, the mean HIC levels were significantly elevated in both strains of mice fed with the ferrocene-diet. The elevation in WT group was about 7.2-fold higher than control (0.353±0.281 to 2.574±1.019 mg/g dry weight, p <0.0001). After two months of CUR treatments, a slight lowering in HIC in WT mice was seen (which did not reach statistical significance). In BKO mice that received ferrocene the HIC was about 6.2 times higher than control



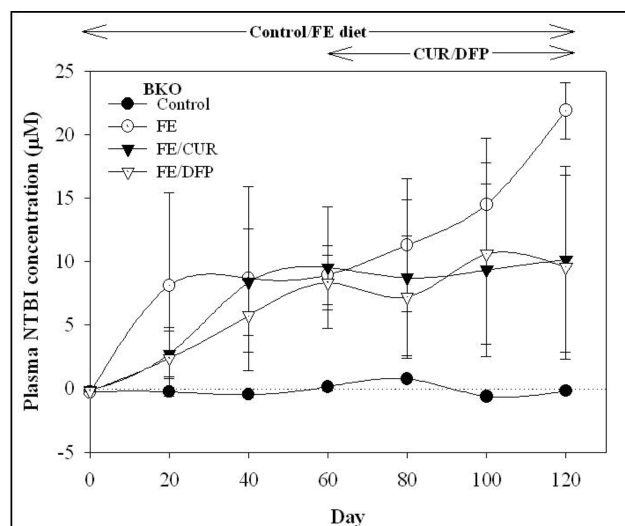


Fig. (3). Plasma non-transferrin bound iron (NTBI) concentrations of the WT mice (top) and BKO mice (bottom) fed with control and FE diets, together with CUR and DFP treatments. Data are expressed as mean±SD (n = 12).

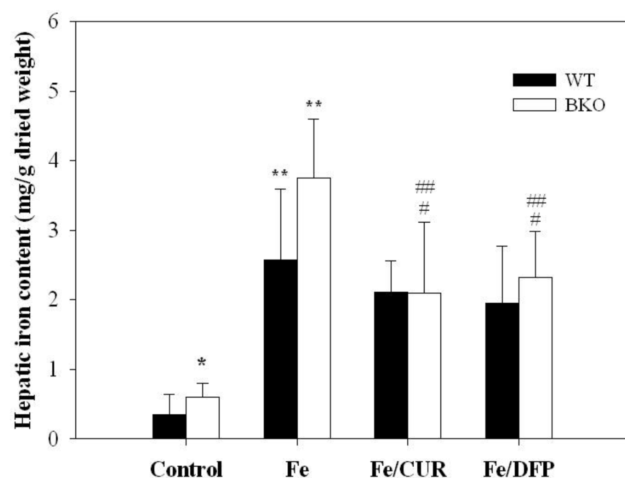


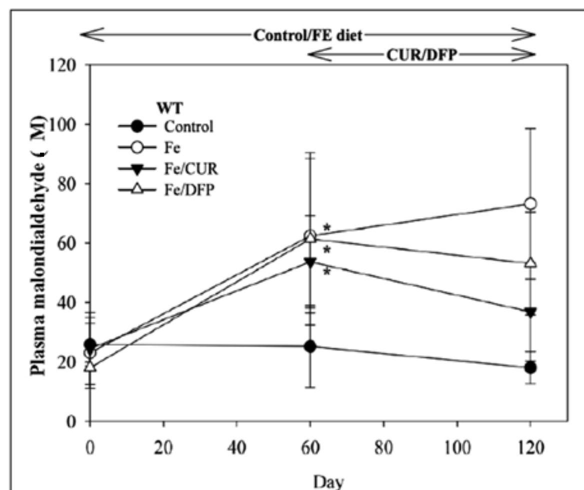
Fig. (4). Hepatic iron concentrations of the WT and BKO mice fed with control and FE diets, together with CUR and DFP treatments. Data are expressed as mean±SD (n = 12). $^{*}p$ <0.001 compared with WT control diet, $^{**}p$ <0.0001 compared with control diet, $^{\#}p$ <0.001 and $^{\#\#}p$ <0.01 compared with FE diet.

(0.605±0.118 to 3.751±0.851 mg/g dry weight, p <0.001) and, treatment with CUR significantly lowered nonheme liver iron (from 3.751±0.851 to 2.104±0.460 mg/g dry weight, p <0.01). DFP also significantly lowered LIC in and BKO mice but this did not reach significance with WT mice at this dose. Taken with the NTBI results, these findings suggest that CUR can chelate excess iron, either in form of NTBI or directly in the liver.

Plasma and Hepatic Lipid Peroxidation

One mechanism proposed for liver injury resulting from iron overload is increased lipid peroxidation of macromolecules, especially lipid, catalyzed by cellular low-molecular weight labile iron pools. In this study, malondialdehyde was measured over the course of administration period as an index of lipid peroxidation in plasma and liver homogenates. As demonstrated in Fig. (**5**), dietary iron administration to WT mice produced significant levels of increased plasma MDA compared with control group (p < 0.01). It was found that intervention with CUR for 60 days abolished such plasma lipid peroxidation (53.67±15.50 to 36.85±16.56 µM). In BKO mice, the ferrocene supplemented diet also significantly elevated plasma MDA concentration (p <0.0001). Two-months of treatment with CUR resulted in a significant decrease of lipid peroxidation (65.12±14.68 to 44.97±12.45 µM, p <0.01). The trend for increasing plasma MDA levels with time in the BKO mice receiving a standard diet is consistent with increased GI iron absorption and the hepatic iron accumulation as seen in Fig. (**4**), secondary to ineffective erythropoiesis.

The Lipid-peroxidation product, MDA, was also measured in liver homogenates using a HPLC-based analysis. Consistent with the rise of plasma MDA (Fig. (**5**)) and HIC (Fig. (**4**)), mean hepatic MDA levels in the iron treatment groups were also significantly elevated in both WT (0.419± 0.333 to 2.309±0.166 µmole/mg protein, p <0.01) and BKO



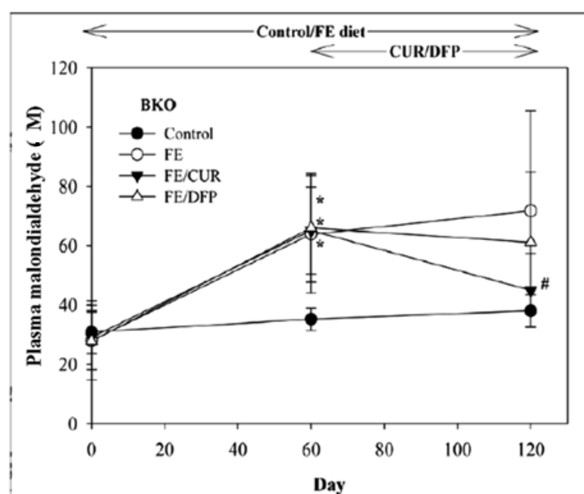


Fig. (5). Plasma malondialdehyde (MDA) concentrations of WT mice (top) and BKO mice (bottom) fed with control and FE diets, together with CUR and DFP treatments. Data are expressed as mean±SD (n = 12). $^{*}p$ <0.01 compared with control diet, $^{\#}p$ <0.01 compared with values on day 60.

(1.234±0.798 to 2.514±0.379 µmole/mg protein, p = 0.002) mice. Levels of hepatic MDA were decreased about 1.9- and 1.3-fold in mice receiving CUR and DFP, respectively (Fig. (**6**)). The decreased lipid peroxidation with CUR may result from the binding and removal of iron but also possibly from the direct scavenging of free radicals.

Liver Glutathione Level

To assess effects of CUR on antioxidative status in iron-loaded mice, we investigated total glutathione levels in liver homogenates. At baseline, glutathione level in liver homogenates of thalassemic mice was markedly lowered than WT mice (p <0.001). As seen in Fig. (**7**), ferrocene supplemented diet did not alter liver total glutathione level in WT mice. The level tended to increase after intervention with CUR for 60 days. By contrast, iron supplemented diets significantly suppressed GSH level in liver homogenates of

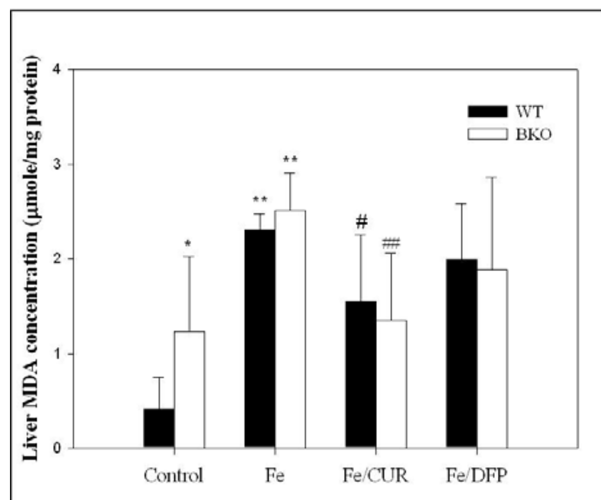


Fig. (6). Liver MDA concentrations of WT and BKO mice fed with control and FE diets, together with CUR and DFP treatments. Data are expressed as mean±SD (n = 12). $^{*}p$ <0.0001 compared with WT control diet, $^{**}p$ <0.01 compared with control diet, $^{\#}p$ <0.01 and $^{\#\#}p$ <0.05 compared with FE diet.

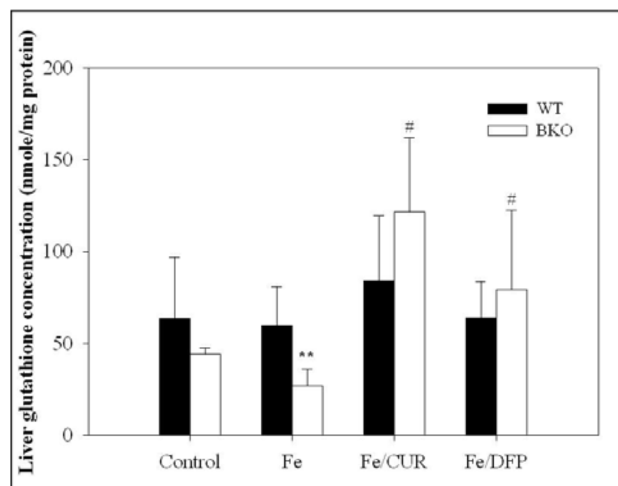


Fig. (7). Liver total glutathione concentrations of WT and BKO mice fed with control and FE diet, together with CUR and DFP treatments. Data are expressed as mean±SD (n = 12). $^{**}p$ <0.001 compared with control diet, $^{\#}p$ <0.01 compared with FE diet.

BKO mice (p = 0.001). Interestingly, GSH levels were significantly elevated in CUR-treated mice versus iron-loaded mice (p <0.01). Similarly, DFP, a known iron chelator, increased the liver GSH concentration, but less effectively than CUR.

Organ Weight Index

Weight parameters have been summarized in Table **1**. Liver, heart and spleen weights were measured as a ratio to total body weight × 100, called weight index. Consistent with previous study, there was no significant difference in the weight indices for liver and heart between WT [25]. However, a highly statistically significant increase (p <0.01) was found in spleen weight index of BKO compared with WT mice, presumably due to increased erythropoietic activity. The mean weight indexes of liver and heart of the iron-loaded WT and BKO mice were significantly increased compared to their respective controls in all treatment groups (p <0.05). In addition, liver and heart weight indices in iron-treated BKO mice were slightly higher than those in iron-treated WT mice. This might be due to iron accumulation in these organs in iron loaded mice or to increased fibrosis tissue. After supplemented with CUR, liver weight index was slightly decreased compared to iron-loaded mice, whereas, such index in DFP treated mice was slightly increased but not significantly when compared with iron treated group.

Iron Accumulation in Heart, Liver and Spleen

Perls' Prussian blue staining was used to investigate iron deposition in vital tissues. As shown in Fig. (**8**) and Fig. (**9**) ferrocene diet markedly increased iron deposition in vital

Table 1. Weight Index of Heart, Liver and Spleen from WT and BKO Mice Fed with Control and FE Diets, Together with CUR and DFP Treatments. Data are Presented as mean±SD (n = 12). *p <0.01 Compared with WT Control Diet, $^{\#}p$ <0.01 Compared with BKO Control Diet

Group \ Parameter	Age Range (Days)	Weight Index (%)		
		Heart	Liver	Spleen
WT control diet	220-258	0.425±0.076	4.477±0.853	0.462±0.346
WT FE diet	220-258	0.529±0.079*	12.355±0.518*	0.559±0.110
WT FE/CUR	220-258	0.538±0.169	12.233±3.106	0.441±0.102
WT FE/DFP	220-258	0.552±0.096	12.574±2.793	0.754±0.394
BKO control diet	226-258	0.413±0.112	5.009±0.096	1.267±0.614*
BKO FE diet	226-258	0.609±0.167#	12.849±2.306#	1.211±0.525
BKO FE/CUR	226-258	0.682±0.154	12.527±2.029	1.474±0.430
BKO FE/DFP	226-258	0.679±0.121	13.834±1.656	1.535±0.199

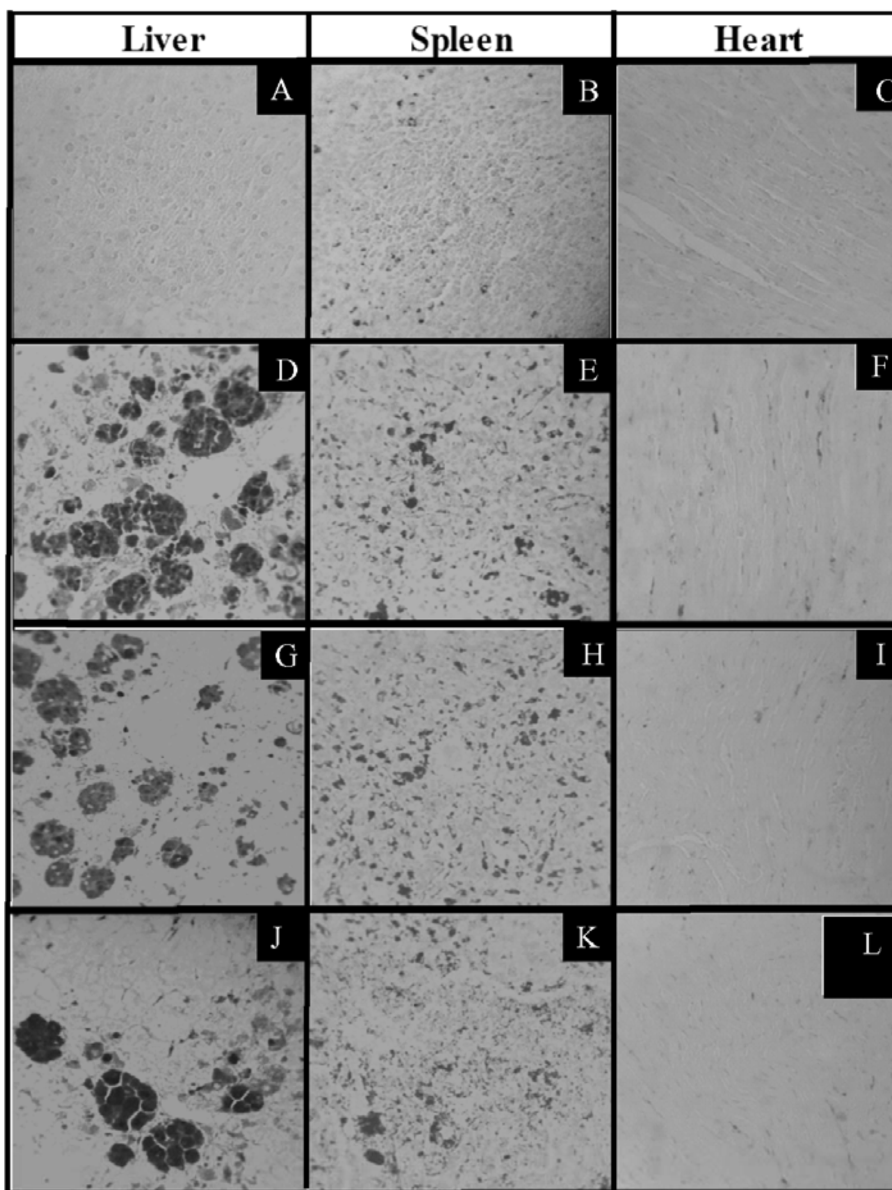


Fig. (8). Perls' Prussian blue staining of liver, spleen and heart of WT mice fed with normal diet (**A**, **B**, **C**) and FE diet (**D**, **E**, **F**), together with CUR treatment (**G**, **H**, **I**) and DFP treatment (**J**, **K**, **L**).

organs especially liver. In comparison, iron accumulation in BKO mice was higher than WT mice. In addition, there was iron accumulation primarily in hepatocytes with only a small amount in Kupffer cells. However, CUR slightly reduced the iron accumulation in both WT and BKO mice.

DISCUSSION

Collectively, these results demonstrate that in the presence of iron overload, CUR has beneficial effects on plasma NTBI, hepatic iron, plasma and liver MDA and on intracellular glutathione. Before discussing these effects in more detail, the animal model used in this study will be discussed briefly. At 3-month of age, there was no iron overload condition in C57BL/6 mice fed on a standard diet (both heterozygous β-knockout and wild type strains). Our study has demonstrated for the first time the induction of severe iron overload in heterozygous β-knockout mice by administration of ferrocene-supplemented diet for 2 months. One of iron overload manifestation is the occurrence of plasma NTBI. In this study, we observed high level of NTBI and MDA in plasma of ferrocene-loaded mice. It could be suggested that the increase of plasma NTBI is responsible for catalyzing reactive oxygen species formation in blood circulation of iron overloaded mice. The resulting radicals, especially hydroxyl radical ($HO^{\bullet}$), potentially oxidize cellular macromolecules and cause various pathologies in iron overloaded diseases. On the other hand increased liver iron levels may themselves lead to increased plasma MDA independently of NTBI. The extent to which the increased hepatic MDA contributes to increased plasma MDA is not clear. It is therefore unclear whether the plasma MDA reflects primarily oxidative events that occur within the plasma compartment (from NTBI in

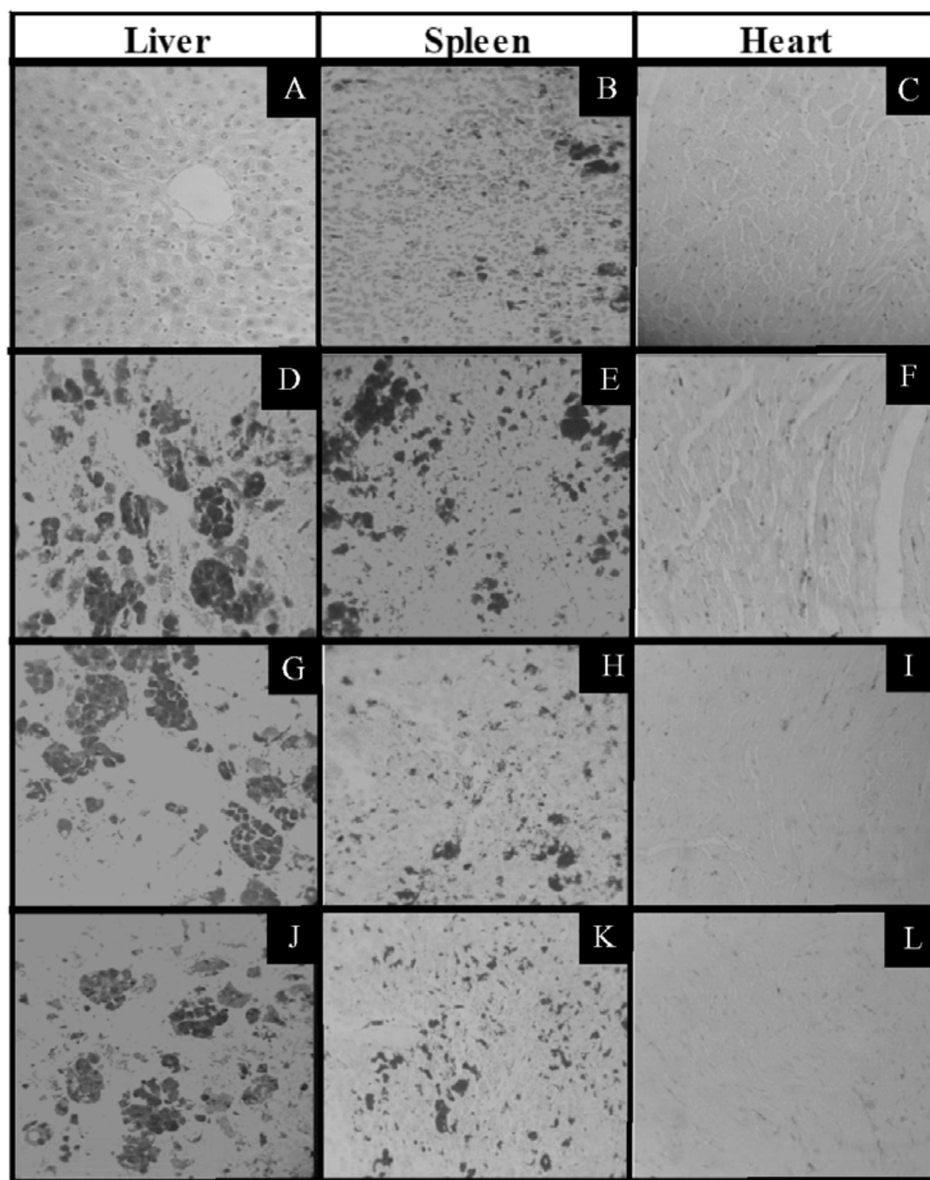


Fig. (9). Perls' Prussian blue staining of liver, spleen and heart of BKO mice fed with control diet (**A**, **B**, **C**) or FE diet (**D**, **E**, **F**), together with CUR treatment (**G**, **H**, **I**) and with DFP treatment (**J**, **K**, **L**).

this case) or whether oxidative events that occur elsewhere, such as the liver, are detected in the plasma as MDA.

The liver is the major organ taking up and storing excess iron [26]; consistently, we found that nonheme iron contents and lipid peroxidation in liver of iron loaded mice were markedly higher than control group. Similarly, *in vivo* studied have reported that lipid peroxidation correlated with the liver iron content of iron overloaded rats [27] and lipid peroxidation has been proposed as an initial step of cellular injury from iron overload [28, 29]. Moreover, Valerio and Petersen reported that administration of iron-supplemented diets led to hepatic fibrosis in mice [30].

Although three iron chelators are in widespread clinical use for the clinical treatment of iron overload a proportion of patients still suffer from the effects of iron overload, either because of ineffectiveness of treatment in individual cases, unacceptable tolerability in some cases, or poor compliance in others. By combining treatments with low toxicity with the above approaches, further benefit may be obtained. There is currently great interest in novel iron chelators and phytochemicals are an attractive option because they often are known to be well tolerated in non clinical use. Curcumin has been previously shown to have low toxicity in both animal and human studies: there was no toxicity in a phase I trial of 25 subjects consuming up to 8000 mg of curcumin per day for 3 months [31]. Another study in humans using 1125–2500 mg of curcumin per day also reported no toxicity [32]. Interestingly, we have previously reported that curcumin increased the rate of NTBI removal from thalassemic plasma, *in vitro* [21].

In this study, we have demonstrated that CUR effectively decrease raised levels of NTBI and HIC in iron-loaded mice, both wild type and heterozygous β-knockout strains. Iron

chelation may occur *via* the β-diketonate group, a known bidentate chelator of Fe^{3+}. Using a Cyclic voltametric assay, the formation constant of Fe^{3+}-curcumin complex was found to be 10^{22} M^{-1} [20]. In this study, CUR supplementation also decreased iron deposition and lipid peroxidation in liver. These effects might be expected to decrease liver damage under iron overload conditions. CUR substantially decreased MDA in both liver and plasma but also boosted the level of total glutathione in liver of iron-loaded mice, especially in the thalassemic mice. Such an ability to protect against oxidative stress is a key requirement of iron overload treatment. While the experiments in the study to not clarify whether the decrease in NTBI with CUR is a direct effect, or is secondary to lowering of body iron, some degree of direct chelation of NTBI by CUR is likely because of our previous studies with plasma taken from thalassemia major patients; showed direct removal of NTBI. In these studies, the beneficial effects of CUR compared favorably with those of DFP. It should be noted however that DFP was administered only once daily at a dose of 50mg/kg which a little less than the standard clinical daily dose of 75mg/kg. In future preclinical studies, it would be of interest to examine the efficacy of CUR in removal of the labile iron pool (LIP) from liver cells and from cardiomyocytes.

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