Original article     http://dx.doi.org/10.1016/j.apjtb.2015.09.007

# Combined treatment of 3-hydroxypyridine-4-one derivatives and green tea extract to induce hepcidin expression in iron-overloaded β-thalassemic mice

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## ARTICLE INFO

## ABSTRACT

**Objective:** To evaluate the efficacy of deferiprone (DFP), 1-(N-acetyl-6-aminohexyl)-3-hydroxy-2-methylpyridin-4-one (CM1) or green tea extract (GTE) in enhancing expression of hepatic hepcidin1 (Hamp1) mRNA and relieving iron overload in β-globin knockout thalassemic mice.
**Methods:** The β-globin knockout thalassemic mice were fed with a ferrocene-supplemented diet for 2 months and oral administration of deionized water, DFP (50 mg/kg), CM1 (50 mg/kg), GTE (50 mg epigallocatechin 3-gallate equivalent/kg), GTE along with DFP (50 mg/kg), and GTE along with CM1 (50 mg/kg) every day for 3 months. Levels of hepatic Hamp1 mRNA, plasma non-transferrin bound iron, plasma alanine aminotransferase activity and tissue iron content were determined.
**Results:** All chelation treatments could reduce plasma non-transferrin bound iron concentrations. Additionally, hepatic Hamp1 mRNA expression was significantly up-regulated in the mice in a GTE + DFP combined treatment, correlating with a decrease in the plasma alanine aminotransferase activity and tissue iron deposition.
**Conclusions:** The GTE + DFP treatment could ameliorate iron overload and liver oxidative damage in non-transfusion dependent β-thalassemic mice, by chelating toxic iron in plasma and tissues, and increasing hepcidin expression to inhibit duodenal iron absorption and iron release from hepatocytes and macrophages in the spleen. There is probably an advantage in giving GTE with DFP when treating patients with iron overload.

## 1. Introduction

Secondary iron overload in β-thalassemia patients is caused by multiple blood transfusions and an increase of duodenal iron absorption [1]. Apparently, toxic forms of iron known as non-transferrin bound iron (NTBI), labile plasma iron and labile iron pools (LIP) are detectable in these patients [2,3]. Effective iron chelators are required to remove this iron to prevent oxidative damage in the vital organs, particularly the heart and liver. Nowadays, deferoxamine (DFO), deferiprone (DFP) and deferasirox (DFX) are the iron chelators which are usually used for the treatment of β-thalassemia patients with iron overload; however, they do produce adverse effects [4]. 1-(N-Acetyl-6-aminohexyl)-3-hydroxy-2-methylpyridin-4-one (CM1) has been synthesized and proposed as a new bidentate iron chelator whose chelating property and toxicity have so far been investigated in vitro and in animals [5–7]. Green tea extract

(GTE) has been reported as a strong antioxidant, as well as a natural iron chelator both *in vitro* and *in vivo* [8–10]. Furthermore, iron chelators have been reported to increase hepatic hepcidin expression [11,12]. Importantly, increased hepcidin expression reduces iron overload and improves anemia effectively [13,14].

Hepcidin, a hepatic 25-amino acid peptide hormone, is synthesized and released into the blood circulation to regulate systemic iron metabolism [15,16]. It inhibits iron flux from enterocytes, hepatocytes and macrophages to the blood stream by binding to the iron-exporter ferroportin causing its internalization and degradation [17,18]. The regulations result in a retention of iron within the cells and a reduction of iron in the plasma [19–21]. Recently, NTBI and hepcidin have been proposed to be novel reliable markers for iron metabolism, especially, iron overload condition [2,22,23]. Urinary and serum hepcidin levels are decreased in β-thalassemia, which exacerbates the condition leading to further iron overload [24]. Expression of hepcidin in patients with iron overload such as β-thalassemia and myelodysplatic syndromes may be suppressed by the growth differentiation factor 15, the twisted gastrulation factor 1, the bone morphogenetic protein-binding endothelial cell precursor-derived regulator and/or the erythroferrone [2,25–28]. Nonetheless, the mechanism of hepcidin regulation under these conditions is still unclear. In the present study, we investigated the expression and benefits of hepcidin in iron-loaded β-globin knockout (BKO) thalassemic mice treated with single and combined iron chelators. Hopefully, iron chelators would lead to a negative iron balance in the body by enhancing hepcidin expression, resulting in lowering duodenal iron absorption and release of the iron from the liver and macrophages in the spleen.

## 2. Materials and methods

### 2.1. Chemicals and reagents

3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p′*-disulfonic acid monosodium salt hydrate (ferrozine), *bis*-(η5-cyclopentadienyl)-iron (ferrocene), ferrous ammonium sulfate, 3-[*N*-morpholino]propanesulfonic acid, nitrolotriacetic acid trisodium salt (NTA), sodium acetate trihydrate, sodium dodecylsulphate, thioglycolic acid and trichloroacetic acid were purchased from Sigma–Aldrich Chemicals Co. Ltd., St. Louis, MO, USA. TRIzol reagent was purchased from Invitrogen Company, UK. RNA isolation kit (Illustra RNAspin mini RNA isolation kit) was purchased from GE Healthcare Company, UK. High capacity cDNA reverse transcription kit was purchased from Applied Biosystems Company, UK. The EXPRESS SYBR® GreenER™ qPCR Supermix universal kit was obtained from Invitrogen Company, UK. Alanine aminotransferase (ALT) assay kit was purchased from Biotech Co. Ltd., Thailand. Acetonitrile [high performance liquid chromatography (HPLC) grade, density = 0.782 $g/cm^3$] was purchased from BDH, UK.

### 2.2. Iron chelators

DFP was kindly donated by the Government Pharmaceutical Organization of Thailand. CM1 was synthesized by Dr. Kanjana Pangjit, Ubon Ratchathani University, Thailand [5]. 1-Methyl-2-propyl-3-hydroxypyridine-4-one (CP22) was kindly donated by

Professor Robert C. Hider, Institute of Pharmaceutical Science, King's College London, United Kingdom.

### 2.3. GTE

Fresh tea (*Camellia sinensis*) leaves were harvested from a local tea plantation in Chiang Mai, Thailand and immediately dried in a microwave cabinet [8]. Hot water crude extract of green tea was prepared and epigallocatechin 3-gallate (EGCG) content was determined by using HPLC method [9,10]. The GTE product containing 24% (w/w) EGCG was kept in the dark at −20 °C until studied.

### 2.4. Animals

Male and female C57/BL6 mice of wild type (WT, $^{mu}β^{+/+}$), aged 2–3 months old and weighed 20–25 g, and heterozygous BKO ($^{mu}β^{th−3/+}$) mice, were bred and supplied by the Thalassemia Research Center, Institute of Molecular Bioscience, Mahidol University, Salaya Campus, Thailand [29,30]. The experimental protocol was conducted with the approval of the Animal Ethical Committee of Medical Faculty, Chiang Mai University, Thailand (Reference No. 42/2556). WT and BKO mice (*n* = 10; 5 in each gender) fed with a CP 082 normal chow diet (N diet) (Perfect Companion Group Co. Ltd., Samuthprakarn, Thailand) were observed as the normal diet control. For iron loading, six groups of BKO mice (*n* = 10; 5 in each gender) were fed with a 0.2% (w/w) ferrocene-supplemented diet (Fe diet) for 2 months (Day 0–60) [31]. On the 60th day, tail vein blood samples were collected before the chelation treatments for analysis of plasma NTBI concentrations and ALT activity. Afterwards, deionized water (DI), DFP (50 mg/kg), CM1 (50 mg/kg), GTE (50 mg EGCG equivalent/kg), GTE (50 mg EGCG equivalent/kg) together with DFP (50 mg/kg), and the GTE (50 mg EGCG equivalent/kg) together with CM1 (50 mg/kg) were orally administered by using a gavage needle to the mice every day for 3 months (Day 61–150) [10]. On the 150th day, all mice were sacrificed and their blood was collected through cardiac puncture into Na-heparin tubes for analysis of plasma NTBI concentrations and ALT activity. The liver, spleen and duodenum were collected, weighed and used for evaluation of the tissue iron by using histochemical Perl's Prussian blue staining technique and ferrozine colorimetric method. Organ weight index (OWI) was calculated with the following formula:

OWI (%) = organ weight (g) × 100/body weight (g)

### 2.5. Quantification of hepatic hepcidin1 (Hamp1) mRNA

RNA was extracted from 100 mg of mouse liver by using TRIzol reagent, and the genomic DNA was removed from the RNA samples by using the RNA isolation kit according to the manufacturer's protocol. A total of 1 μg of RNA was reversely transcribed into cDNA by using a high capacity cDNA reverse transcription kit. Levels of *Hamp1* mRNA in the liver were quantified by using the quantitative real-time PCR (qPCR) with ΔΔCT method [32]. The housekeeping RNA β-actin (*Actb*) was used as an endogenous control to normalize the cDNA samples for relative quantitation. The qPCR reaction of cDNA was performed by using the EXPRESS SYBR® GreenER™ qPCR

Supermix universal kit on the ABI 7500 real-time PCR instrument (Applied Biosystems, UK). The primer sequences used in qPCR were presented as follows: *mHamp1* forward: CCTGAGCAG-CACCACCTATC, *mHamp1* reverse: TGCAACAGATACCA-CACTGGG, *mActb* forward: GGTCCACACCCGCCAC, and *mActb* reverse: GTCCTTCTGACCCATTCCCA. Relative mRNA expression in WT and BKO mice was acquired by normalizing *Hamp1* mRNA to *Actb* mRNA.

## 2.6. Plasma ALT activity assay

Plasma ALT activity of the mice on Day 60 and Day 150 was examined by using ALT assay kit [33]. Difference in the ALT activity was calculated.

## 2.7. Quantification of plasma NTBI

Plasma NTBI concentrations of the mice on Day 60 and Day 150 were measured by using the HPLC method [34]. Briefly, plasma was incubated with a weak chelator NTA solution (at a final concentration of 80 mmol/L, pH 7.0) for 30 min at room temperature to produce $Fe^{3+}$-$(NTA)_2$ complex. Subsequently, the complex was filtered through a membrane (NanoSep®, 10-kDa cut-off, polysulfone type; Pall Life Sciences, USA) and analyzed by using the non-metallic HPLC system.

The $Fe^{3+}$-$(NTA)_2$ representing NTBI was fractionated on a glass analytical column (ChromSep-ODS1, 100 mm × 3 mm, 5 μm particle size) and eluted at a flow rate of 1 mL/min with a mobile phase solvent containing 3 mmol/L CP22 in 19% acetonitrile buffered with 5 mmol/L 3-[*N*-morpholino]propane-sulfonic acid (pH 7.0) to generate a $Fe^{3+}$-$(CP22)_3$ product. Eluents were monitored and detected at 450 nm with a flow cell detector (SpecMonitor2300; LDC Milton–Roy Inc., USA). Data analysis was manipulated by BDS software (BarSpec Ltd., Israel). NTBI concentrations were represented by the $Fe^{3+}$-$(CP22)_3$, while the peak height was determined from a standard curve which was constructed from 0 to 16 μmol/L $Fe^{3+}$-$(NTA)_2$ in 80 mmol/L NTA. The difference in the NTBI concentrations was calculated.

## 2.8. Histochemical examination of tissue iron

Liver, spleen and duodenum tissues were fixed in 10% neutralized formalin. Fixed tissue sections were dehydrated with a gradual series of ethanol, embedded in paraffin, sectioned, and stained with potassium ferrocyanide solution (known as Perl's supravital dye) by using the standard protocol. The stained slides were analyzed under a light microscope by an expert pathologist and photographed with a digital camera.

## 2.9. Determination of tissue iron content (TIC)

TIC was measured by the ferrozine colorimetric method [35]. The liver, spleen and duodenum were dried at 120 °C overnight in a hot air oven. The dried organs were weighed and homogenized in 0.5% (w/v) sodium dodecylsulphate solution. The homogenate was added to the protein precipitating agent (1 mol/L HCl/10% trichloroacetic acid solution), mixed vigorously, and heated at 95 °C for 1 h. After cooled down to room temperature, the protein-precipitated solution was

centrifuged at 12000 r/min for 10 min. Iron in the supernatant was then allowed to react with the chromogenic solution containing 0.508 mmol/L ferrozine, 1.5 mol/L sodium acetate and 1.5% (v/v) thioglycolic acid for 30 min to generate the colored product. The optical density of the product was measured photometrically at 562 nm. Iron concentrations were determined from a calibration curve of 0–200 μmol/L ferrous ammonium sulfate. The TIC was presented as mg/g organ dry weight.

## 2.10. Statistical analysis

Data were analyzed by using the IBM SPSS Statistic 20 program and presented as mean ± SD. Statistical significance was determined by using One-way ANOVA test, and the results with $P < 0.05$ were considered significant.

# 3. Results

## 3.1. OWI

As shown in Table 1, the OWI value of the spleen from the BKO-N diet mice was significantly increased when compared with the WT-N diet mice, and those of the liver and spleen from the BKO-Fe diet mice were significantly elevated when compared with the BKO-N diet mice. However, the chelators did not significantly change any OWI values in the treated BKO-Fe diet mice when compared with the untreated mice.

## 3.2. Hepatic Hamp1 mRNA expression

Hepatic *Hamp1* mRNA expression was not found to be significantly different between genders in both WT and BKO mice (Figure 1). Importantly, the hepatic *Hamp1* mRNA levels in the BKO-N diet mice were significantly lower than those in the WT-N diet mice, while the hepatic *Hamp1* mRNA expression was increased in the BKO-Fe diet mice when compared with the BKO-N diet mice ($P < 0.05$) (Figure 2). The hepatic *Hamp1* mRNA levels were increased significantly in the BKO-Fe diet/GTE + DFP group when compared with the BKO-Fe diet/DI group. Nevertheless, there was no significant change in *Hamp1* mRNA levels in the BKO-Fe diet/DFP, BKO-Fe diet/CM1, BKO-Fe diet/GTE and BKO-Fe diet/GTE + CM1 groups (Figure 2).

**Table 1**

OWI of WT and BKO mice (n = 10, 5 in each gender) fed with N diet or Fe diet along with chelation treatments for 90 days. %.

| Mice/treatment | OWI | |
| --- | --- | --- |
| | Liver | Spleen |
| WT-N diet/DI | 4.73 ± 0.50 | 0.33 ± 0.14 |
| BKO-N diet/DI | 5.03 ± 0.61 | 1.60 ± 0.40[#] |
| BKO-Fe diet/DI | 10.00 ± 0.93[*] | 2.12 ± 0.57[*] |
| BKO-Fe diet/DFP | 9.72 ± 0.91 | 2.05 ± 0.35 |
| BKO-Fe diet/CM1 | 10.06 ± 0.74 | 2.24 ± 0.40 |
| BKO-Fe diet/GTE | 9.73 ± 0.40 | 2.21 ± 0.36 |
| BKO-Fe diet/GTE + DFP | 9.77 ± 0.67 | 2.15 ± 0.23 |
| BKO-Fe diet/GTE + CM1 | 9.54 ± 0.95 | 2.11 ± 0.53 |

[#]: $P < 0.05$ when compared with the WT-N diet/DI group; [*]: $P < 0.05$ when compared with the BKO-N diet/DI group.
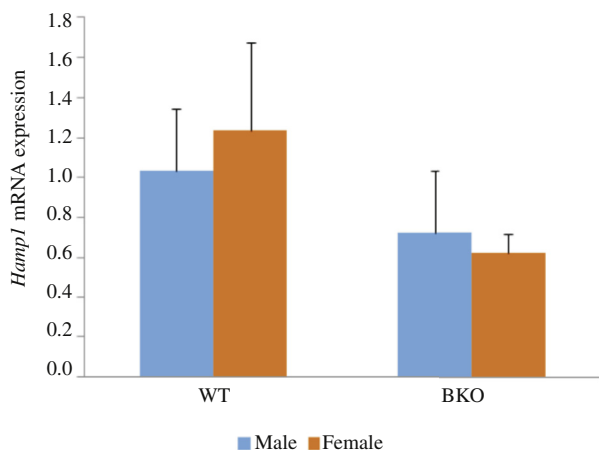
**Figure 1.** qPCR of *Hamp1* mRNA acquired from the livers of WT and BKO mice (*n* = 10, 5 in each gender).
Values are expressed as mean ± SD for the fold change for the comparison between both genders.

### 3.3. Plasma ALT activity

The levels of plasma ALT activity in the BKO-N diet mice were not significantly different from those in the WT-N diet mice (Table 2). However, the levels of plasma ALT activity were increased significantly in the BKO-Fe diet mice when compared with the BKO-N diet mice, implying iron loaded-oxidative liver damage in the BKO-Fe diet mice.

All single chelation treatments tended to lower the increased plasma ALT activity levels with efficacy in the order of DFP > CM1, GTE when compared with the non-treatment group (BKO-Fe diet/DI). Most importantly, the GTE + DFP treatment (mean difference = 1.16 IU/L), but not the GTE + CM1 treatment (mean difference = 20.85 IU/L), was significantly more effective than DFP treatment alone (mean difference = 6.31 IU/L) as well as GTE treatment (mean difference = 11.49 IU/L) and was the most effective in decreasing plasma ALT activity when compared with the non-treatment group.

Expectedly, plasma NTBI levels in the BKO-Fe diet mice were much higher than those in the BKO-N diet mice ($P < 0.05$)
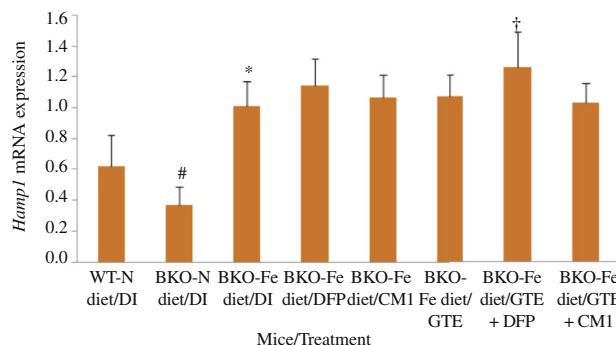
**Table 2**
Plasma ALT activity of WT and BKO mice (*n* = 10, 5 in each gender) fed with the N diet or Fe diet along with chelation treatments for 90 days.

| Mice/treatment | Plasma ALT activity (IU/L) | | |
|---|---|---|---|
| | Day 60 | Day 150 | Difference |
| WT-N diet/DI | 13.65 ± 6.03 | 17.63 ± 3.27 | 3.98 ± 6.66 |
| BKO-N diet/DI | 14.27 ± 5.24 | 16.58 ± 5.29 | 2.31 ± 6.89 |
| BKO-Fe diet/DI | 38.02 ± 11.06[*] | 54.76 ± 12.39[*] | 16.74 ± 11.87[*] |
| BKO-Fe diet/DFP | 35.91 ± 15.21 | 42.22 ± 11.95[†] | 6.31 ± 16.27 |
| BKO-Fe diet/CM1 | 38.29 ± 8.50 | 49.70 ± 10.03 | 11.40 ± 13.22 |
| BKO-Fe diet/GTE | 38.82 ± 10.30 | 50.31 ± 12.20 | 11.49 ± 9.00 |
| BKO-Fe diet/ GTE + DFP | 43.49 ± 17.55 | 44.65 ± 8.30[†] | 1.16 ± 20.11[†] |
| BKO-Fe diet/ GTE + CM1 | 35.95 ± 11.46 | 56.80 ± 11.84 | 20.85 ± 19.11 |

Data are expressed as mean ± SD; [*]: $P < 0.05$ when compared with the BKO-N diet/DI group; [†]: $P < 0.05$ when compared with the BKO-Fe diet/DI group.

(Table 3). All treatments reduced the plasma NTBI levels ($P < 0.05$); however, the GTE + DFP treatment seemed to show the greatest effect.

Perl's Prussian blue staining results (Figure 3) revealed numerous hemosiderin in sinusoidal macrophages, hepatocytes and splenic macrophages in both red and white pulps of the BKO-Fe diet mice. The duodenum also showed iron accumulation with rare hemosiderin-laden macrophages in mucosa when compared with the BKO-N diet mice. The WT-N diet mice had no iron accumulation, and the BKO-N diet mice showed the iron accumulation in the spleen with scattered hemosiderin-laden macrophages in both red and white pulps. Only the GTE + DFP combined treatment group showed less iron accumulation in the liver and spleen, as compared with the BKO-Fe diet group, but there was no iron accumulation in the duodenum. Consistently, results of TIC demonstrated that the BKO-Fe diet mice had significant iron accumulation in the liver, spleen and duodenum when compared with the BKO-N diet mice (Table 4). All chelation treatments reduced the iron deposition slightly in the liver and spleen, but not in the duodenum.

Obviously, the GTE + DFP combined treatment had the greatest effect in decreasing the iron deposition significantly in the liver and spleen.



**Figure 2.** Effect of the chelation treatments on hepatic *Hamp1* mRNA expression in the N diet and Fe diet-fed WT and BKO mice for 90 days (*n* = 10, 5 in each gender).
Values are expressed as mean ± SD for the fold change for the comparison with the BKO-Fe diet/DI group. [#]: $P < 0.05$ when compared with the WT-N diet/DI group; [*]: $P < 0.05$ when compared with the BKO-N diet/DI group; [†]: $P < 0.05$ when compared with the BKO-Fe diet/DI group.

**Table 3**
Plasma NTBI concentrations of WT and BKO mice (*n* = 10, 5 in each gender) fed with the N diet or Fe diet along with chelation treatments for 90 days.

| Mice/treatment | Plasma NTBI concentrations (µmol/L) | | |
|---|---|---|---|
| | Day 60 | Day 150 | Difference |
| WT-N diet/DI | 1.20 ± 0.48 | 1.20 ± 0.26 | 0.00 ± 0.33 |
| BKO-N diet/DI | 1.00 ± 0.43 | 1.00 ± 0.35 | 0.00 ± 0.50 |
| BKO-Fe diet/DI | 4.14 ± 1.70[*] | 14.71 ± 1.21[*] | 10.57 ± 2.14[*] |
| BKO-Fe diet/DFP | 6.48 ± 3.95[†] | 9.17 ± 3.12[†] | 2.69 ± 5.47[†] |
| BKO-Fe diet/CM1 | 5.51 ± 1.97 | 9.22 ± 3.19[†] | 3.71 ± 3.09[†] |
| BKO-Fe diet/GTE | 5.54 ± 1.98 | 9.02 ± 3.11[†] | 3.47 ± 2.96[†] |
| BKO-Fe diet/ GTE + DFP | 4.75 ± 0.90 | 6.77 ± 2.68[†] | 2.02 ± 2.96[†] |
| BKO-Fe diet/ GTE + CM1 | 4.13 ± 0.90 | 7.91 ± 3.51[†] | 3.78 ± 3.14[†] |

Data are expressed as mean ± SD. [*]: $P < 0.05$ when compared with the BKO-N diet/DI group; [†]: $P < 0.05$ when compared with the BKO-Fe diet/DI group.
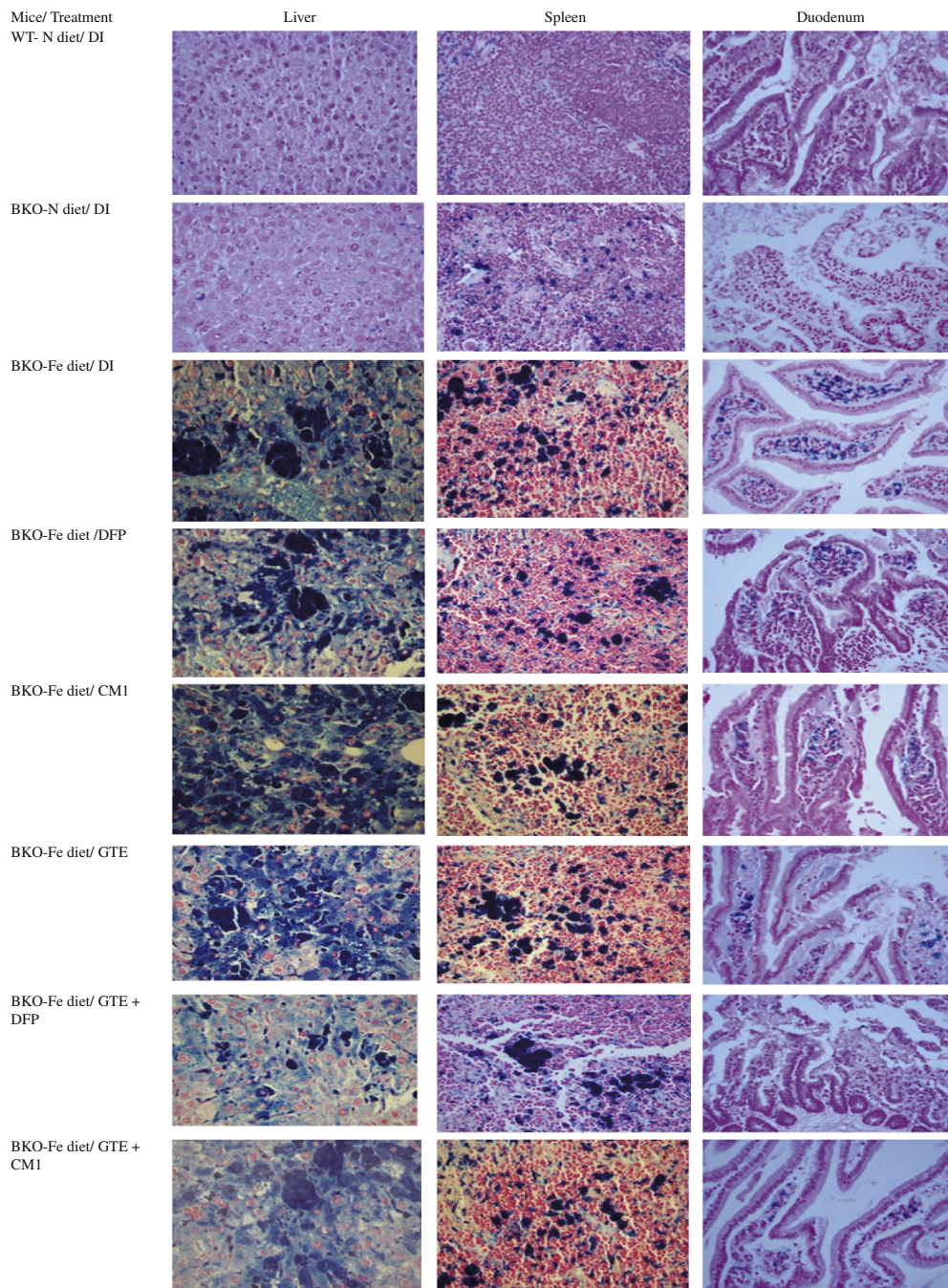
**Figure 3.** Perl's Prussian blue stained tissues of the N diet or Fe diet-fed WT and BKO mice along with the chelation treatments for 90 days.

TIC of WT and BKO mice ($n$ = 10, 5 in each gender) fed with the N diet or Fe diet along with chelation treatments for 90 days.

| Mice/treatment | TIC (mg/g dry weight) | | |
|---|---|---|---|
| | Liver | Spleen | Duodenum |
| WT-N diet/DI | 0.18 ± 0.10 | 1.86 ± 1.03 | 0.14 ± 0.05 |
| BKO-N diet/DI | 0.52 ± 0.25 | 3.06 ± 0.61 | 0.17 ± 0.09 |
| BKO-Fe diet/DI | 19.46 ± 1.62* | 9.30 ± 1.98* | 0.43 ± 0.09* |
| BKO-Fe diet/DFP | 16.85 ± 4.22 | 9.07 ± 2.51 | 0.41 ± 0.13 |
| BKO-Fe diet/CM1 | 17.10 ± 5.96 | 8.82 ± 3.22 | 0.41 ± 0.14 |
| BKO-Fe diet/GTE | 17.67 ± 3.41 | 8.30 ± 1.41 | 0.41 ± 0.07 |
| BKO-Fe diet/ GTE + DFP | 14.77 ± 2.16† | 7.65 ± 1.02† | 0.36 ± 0.10 |
| BKO-Fe diet/ GTE + CM1 | 17.27 ± 5.37 | 8.39 ± 1.26 | 0.48 ± 0.13 |

Data are expressed as mean ± SD. *: $P < 0.05$ when compared with the BKO-N diet/DI group; †: $P < 0.05$ when compared with the BKO-Fe diet/DI group.

## 4. Discussion

We used the BKO thalassemic mice loaded with ferrocene to imitate iron overload in β-thalassemia intermediate patients. The BKO mice displayed ineffective erythropoiesis, mild anemia and splenic enlargement [29,36]. Plasma ALT activity, plasma NTBI concentrations and liver iron accumulation in the BKO-N diet mice were not much different from those in the WT-N diet mice, whereas levels of these parameters were considerably increased in the BKO-Fe diet mice. Some other groups have demonstrated that mRNA expressions of iron-regulatory proteins such as hepcidin, ferroportin, and transferrin receptors vary between the genders of the mice [37,38]. However, we did not find any difference in hepatic *Hamp1* mRNA expression between male and female of WT and BKO mice in this study. That is in accordance with the hepcidin expression in Huh7 cells grown in the condition containing male and female β-thalassemic

patient sera (Upanan S, unpublished data). Therefore, the gender of the mice does not appear to affect basal hepcidin expression in their livers.

We found that BKO mice had significantly lower *Hamp1* mRNA levels than WT mice, which was consistent with previous reports in β-thalassemic mice and humans [39,40]. Interestingly, the up-regulation of *Hamp1* mRNA expression in the BKO-Fe diet mice found in this study would reflect the increase of their tissue iron stores or serum iron concentrations in order to suppress duodenal iron absorption and iron release from the liver and macrophages in the spleen. Gardenghi *et al.* also reported that levels of hepcidin were increased while iron concentrations in the organs were increased and hepcidin still partially responded to iron overload in β-thalassemic mice [41]. The levels of iron-loading in this study may overcome the effects of hepcidin suppressors, resulting in hepcidin up-regulation. Controversially, iron levels might not increase hepcidin expression in β-thalassemia with ineffective erythropoiesis according to high levels of hepcidin suppressors (*e.g.*, growth differentiation factor 15, twisted gastrulation factor 1, bone morphogenetic protein-binding endothelial cell precursor-derived regulator or erythroferrone), which do have a greater influence on decreasing hepcidin levels [27,42,43]. However, the increased hepcidin levels in the GTE + DFP treatment could not result from the iron-loading effect with regard to the reduction of the plasma NTBI levels and iron accumulation in the tissues when compared with the non-treatment group.

Accordingly, GTE + DFP combined treatment was more effective than the DFP treatment alone in diminishing iron overloaded liver damage and consequently decreasing plasma levels of ALT activity in the BKO-Fe diet mice. We expect that green tea catechins may potentiate iron depletion in cooperation with DFP chelator by shuttling the iron from the $Fe^{3+}$-$(DFP)_3$ complex. When DFP and DFO co-exist in plasma, DFP would firstly shuttle intracellular iron and plasma NTBI and transfer them to DFO to form a ferrioxamine, implying that DFP commits to removing labile iron in the liver cells [44,45]. Similarly, the GTE catechins (particularly EGCG) could enhance DFP chelation by shuttling the iron from the iron-DFP complex to form the iron-EGCG complex [9,10]. Significantly, the GTE + DFP combined treatment lowers levels of plasma ALT activity and liver iron accumulation efficiently, suggesting that green tea polyphenols would improve iron-induced dysfunction and injury of the livers in BKO-Fe diet mice. A current study has supported that the combined chelation treatment synergized mobilization of labile iron pools in Huh7 cells, in which the DFX + DFP treatment was the most effective [45]. Though effect of green tea consumption on iron absorption is not conclusive definitely, we believe that it will not affect our study [46,47].

Consistently, green tea significantly increased glutathione peroxidase, catalase, quinine reductase and glutathione *S*-transferase activities in the liver and improved the liver function by its anti-oxidative effect against hepatotoxicity [48–50]. Taken together, this may explain the increased hepatic hepcidin expression following the improvement of the liver functions. In addition, DFX chelation treatment could decrease plasma ALT levels in the patients with iron overload-associated liver dysfunction, and elevate serum hepcidin level in iron-overloaded patients with myelodysplastic syndrome [51]. Unexpectedly, GTE + CM1 combined treatment neither induced hepcidin

expression nor reduced the plasma ALT activity. Since CM1 molecule is more lipophilic, but bigger than DFP, the GTE + CM1 combined treatment may be less efficient than the GTE + DFP combined treatment in removing the iron via the proposed iron-shuttling mechanism. In all likelihood as mentioned above, we imply that the GTE + DFP combined treatment is more effective than the GTE + CM1 combined treatment and the single chelation treatments in the reduction of plasma NTBI concentrations and iron accumulation in the liver and spleen. Consequently, the GTE + DFP treatment could relieve oxidative damage in the liver and enhance hepcidin expression and secretion, and the latter will limit iron absorption in the duodenum and iron release from hepatocytes and macrophages in the spleen.

In conclusion, GTE + DFP combined treatment is the most effective in increasing *Hamp1* mRNA expression and reveals beneficial health effects by lowering plasma NTBI levels, tissue iron deposit and liver oxidative damage in β-thalassemic mice with iron overload. Efficacy of GTE + DFP combined treatment on hepcidin expression/secretion and changes of iron parameters should be further investigated clinically in β-thalassemia patients with iron overload.

## Conflict of interest statement

We declare that we have no conflict of interest.

## Acknowledgments

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