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ORIGINAL RESEARCH

Deferiprone, an iron chelator, alleviates platelet hyperactivity in patients with β-thalassaemia/HbE

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Abstract

Background: Hyperfunctional platelets play important roles in thromboembolism in patients with β-thalassaemia/haemoglobin E (β-thal/HbE). Our previous study revealed ex vivo inhibitory effects of deferiprone on normal platelets. Herein, we aimed to investigate the in vivo effects on platelets in patients with β-thal/HbE.

Methods: A prospective, self-controlled clinical study on 30 patients with β-thal/HbE who had received therapeutic deferiprone (20.8–94.5 mg/kg/day) was conducted. The study included a 4-week washout period followed by 4 and 12 weeks of deferiprone treatment. Platelet aggregation was performed by a turbidimetric method. Levels of deferiprone and soluble platelet (sP)-selectin in serum were measured by high-performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA) kit, respectively.

Results: The washout period significantly enhanced platelet hyperactivity both in patients who had undergone splenectomy and in those who had not. At 2 hours following the administration of a single dose of deferiprone, platelet sensitivity to ADP and arachidonic acid was significantly reduced. The inhibitory effects of deferiprone were gradually increased over the period of 4 and 12 weeks. Deferiprone also depressed sP-selectin levels, but the effect was stable over longer follow-up periods. Correlation analysis demonstrated the relationship between serum levels of deferiprone, sP-selectin, and platelet activities induced by ADP and arachidonic acid.

Conclusion: We first demonstrated the in vivo antiplatelet effect and benefit of short-term treatment of deferiprone in patients with β-thal/HbE. The impact on thrombotic outcomes deserves further study.

Keywords: arachidonic acid, deferiprone, iron overload, platelet aggregation, P-selectin, thalassaemia.

Citation

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Introduction

β-Thalassaemia (β-thal) is a major type of thalassaemia characterized by the absence of or reduction in β-globin chain synthesis. β-Thal/haemoglobin E (HbE) disease, caused by the co-inheritance of β-thal alleles and haemoglobin E variant, is the most common β-thal in Asia, comprising approximately 50% of the clinically severe cases of β-thal.[1,2] The precipitated excess α-globin chain leads to ineffective erythropoiesis and haemolytic anaemia,[3] with the need for regular blood transfusion in patients with severe disease. Together with increased gut iron absorption secondary to ineffective erythropoiesis, most patients develop iron overload in the first decade of life.[4] Iron plays key roles in several complications in β-thal. Iron-induced oxidative stress has

also been associated with platelet hyper-aggregation and is finally involved in pulmonary hypertension and venous thromboembolism.[5–7]

Deferiprone (1,2-dimethyl-3-hydroxypyridin-4-one), a bidentate orally active iron chelator, has been approved in several countries for the treatment of transfusional iron overload in β-thal and, recently, in sickle cell disease. Given that if is a small molecule, deferiprone mobilizes iron from both intracellular and extracellular iron pools.[3,5–9] Viprakasit et al.[10] reported on the effectiveness of deferiprone monotherapy in paediatric patients in Thailand who are transfusion dependent. The benefit of deferiprone over deferoxamine on the removal of myocardial iron has been reported in several studies.[11]

In addition to its iron-chelating ability, the antiplatelet activity of deferiprone has been reported in both in vitro and ex vivo models with platelets from healthy volunteers.[12] Our previous study demonstrated that deferiprone inhibited aggregation induced by ADP, arachidonic acid (AA), epinephrine, and collagen in platelets from healthy volunteers. A higher potency was observed in ADP and AA, with a median effective dose (ED_{50}) of approximately 0.25 mmol/l, whereas the ED_{50} for epinephrine and collagen was 3.4 mmol/l. Moreover, an in silico study demonstrated that deferiprone was able to bind with the active site of COX1, suggesting that inhibition of COX1 may contribute to the antiplatelet activity of deferiprone.[13]

The maximum concentration of deferiprone in β-thal is 0.06–0.15 mmol/l after administration of a single dose of 25 mg/kg.[14] Therefore, therapeutic doses of deferiprone (25–100 mg/kg/day) may inhibit platelet function. However, the inhibitory effects of deferiprone on platelet function in β-thal have not been investigated.

This study was designed to evaluate the antiplatelet effect of deferiprone in patients with transfusion-dependent β-thal/HbE at the dose of iron-chelation therapy. The immediate response and long-term effect of deferiprone was studied at 2 hours after administration and up to 12 weeks of chelation therapy. Our results may provide a mechanistic insight in antiplatelet effects and support the benefit of deferiprone in the prevention of thrombotic events in β-thal.

Methods

Patients

Thirty patients with β-thal/HbE (men or women aged 18–55 years old) treated with deferiprone at 20.8–94.5 mg/kg/day (divided in doses 2–3 times per day) were recruited. Study patients were divided into two groups: those who had undergone splenectomy (splenectomy group) and those who had not (non-splenectomy group). Patients were asked to refrain from any medicine, vitamin, or dietary supplement known to affect platelet function (except daily folic acid) for at least 2 weeks before the first visit and throughout the study period. Exclusion criteria were active hepatitis, neutropenia, thrombocytopenia, hepatic or renal failure requiring treatment, chronic diseases, including heart failure, hypertension or diabetes, and taking antiplatelet or anticoagulant medication. The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Thailand (COA No. 642/2018 and date of approval on July 12, 2018). Informed consent was obtained from all patients involved in the study. Written informed consent was obtained from the patients prior to collecting blood and for publication of this paper.

Study design and intervention

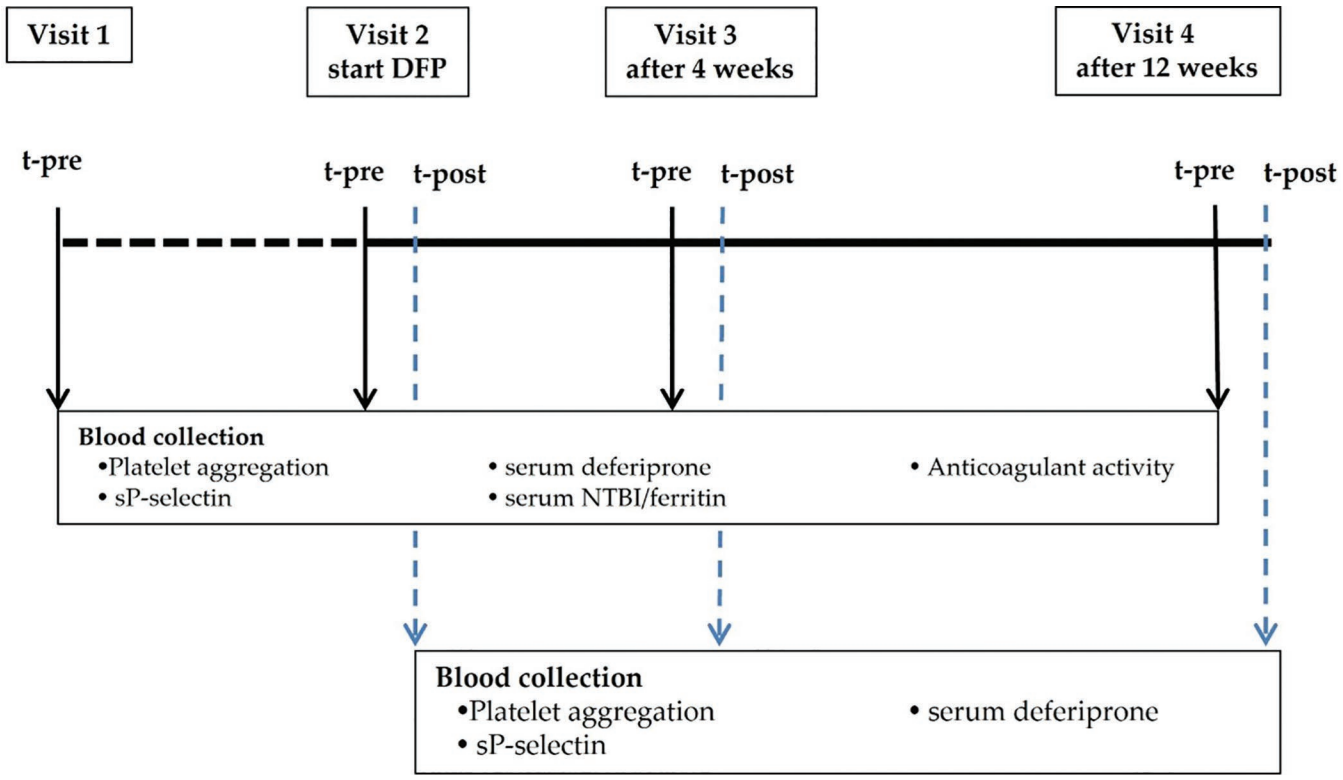
The study was a prospective, self-controlled clinical trial. The study included a washout period of deferiprone for 4 weeks followed by 12 weeks of treatment. After a 4-week washout period, patients were prescribed deferiprone (GPO-L-ONE, Government Pharmaceutical Organization, Thailand) 20.8–94.5 mg/kg divided in 2–3 daily doses, continued for 12 weeks.

Patients attended four visits: baseline (visit 1), after a 4-week washout period (visit 2), and after 4 and 12 weeks of deferiprone treatment (visits 3 and 4). In the morning of each visit, a 20-ml venous blood sample (pre-dosing) was collected and patients were then given a morning oral dose of deferiprone at 9.5–33.0 mg/kg with meals (at visits 2, 3 and 4 only). A 13-ml venous blood sample was obtained from each patient 2 hours after taking deferiprone (post-dosing). In patients who are transfusion dependent, blood samples were obtained prior to red blood cell transfusion at each visit. Blood samples were sent for chemistry analysis and serum and platelet preparation for further assays. The study schedule is summarized in Figure 1.

Iron status

Serum non-transferrin-bound iron (NTBI) levels were determined by a previously described colorimetric method.[15] Briefly, a 450-µl serum sample was mixed with 50 µl of 0.2 mol nitrilotriacetic acid (Sigma Chemical Co., St. Louis, USA), incubated at room temperature for 30 minutes, then ultrafiltered through a Microcon-30 centrifugal filters with Ultracel PL-30 (Merck Millipore, Darmstadt, Germany). A 100-µl filtrate sample was mixed with 25 µl of 150 mmol/l thioglycolic acid and 25 µl of 0.05 mol bathophenanthroline-disulfonic acid (BPT) and incubated at room temperature for 90 minutes. The absorbance of Fe(II)-BPT complex was measured at 537 nm.

Figure 1. Timeline of study. t-pre, a time before the morning dose of deferiprone (DFP) and t-post, 2 hours after the morning dose of deferiprone.



Serum ferritin was determined using an automated chemical analyser as a routine test in clinical settings.

Determination of deferiprone in serum

The level of deferiprone in serum samples was measured by a previously described HPLC method.[14] A 300-µl aliquot of the serum sample was deproteinized by centrifuging at 14,000 *g* for 30 min using an Amicon Centrifree micropartition device (Mw 30,000 cut-offs; Millipore, Bedford, MA, USA) and then a 20-µl aliquot of the filtrate was injected into the reversed-phase HPLC system for the determination of the non-glucuronide deferiprone.

The separation was performed under isocratic conditions on an Eclipse XDB-C18 column (5 µm, 150×4.6 mm internal diameter; Agilent Technologies, Waldbronn, Germany) with a guard column in a Waters 2695 separation module with an autosampler (Waters Corporation, Milford, MA, USA). The eluent was monitored at 280 nm by a Waters 2487 dual absorbance detector. The mobile phase consisted of 10 mmol/l sodium dihydrogen phosphate at pH 3 (adjusted with phosphoric acid), containing 2 mmol/l EDTA and methanol at 93:7 (v/v). The flow rate was 1.0 ml/min. The peak areas were integrated using Millenium 3.2 software (Waters Corporation).

Preparation of platelet and platelet aggregation test

The 3.2% citrate (9:1, v/v) blood samples were centrifuged at 180 *g*, 21°C, for 15 minutes to obtain platelet-rich plasma (PRP) and sediment blood was further centrifuged at 1,500 *g*, 21°C, for 15 minutes to obtain platelet-poor plasma (PPP).[16] Platelet aggregation test was done within 4 hours of blood sample collection and 30 minutes after PRP preparation, and was performed as in our previous studies[13,17] modified from the Born turbidimetric method[18] using an aggregometer (Aggram, Helena Laboratory, Beaumont, TX, USA). Briefly, PRP (225 µl) was incubated at 37°C for 1 minute for baseline and then 25 µl of various concentrations of agonist, ADP or AA were added. The changes in light transmittance at 650 nm were recorded for at least 5 minutes after adding agonists. The response of PRP to each agonist was expressed as ED_{50}.

Determination of sP-selectin

The serum level of soluble P-selectin (sP-selectin) was measured by using a P-selectin human ELISA kit according to the manufacturer's recommendations (Sigma-Aldrich Co., St. Louis, USA). Briefly, P-selectin present in serum samples was bound to the immobilized antibody CD62P coating, then a biotinylated detection antibody

specific for P-selectin was added. After incubation with the HRP-conjugated streptavidin and ELISA colorimetric TMB substrate solution, the intensity of the colour developed in proportion to the amount of P-selectin bound and was measured at 450 nm. The concentration of sP-selectin was determined by interpolation to the standard curve of human P-selectin.

Determination of anticoagulant activity

Anticoagulant activity of deferiprone in vivo was measured by using activated partial thromboplastin time (aPTT) and prothrombin time (PT) assays. Briefly, for aPTT, aPTT reagent (100 µl) was added into 100 µl of PPP and incubated for 1 minute at 37°C, and then 20 mmol/l $CaCl_2$ (100 µl) was added. Clotting time in seconds was recorded. For PT assays, PT assay reagent (200 µl) was added into 100 µl of PPP and incubated for 3 minutes at 37°C; finally, clotting time in seconds was recorded.

Statistical analysis

The Wilcoxon signed rank test was applied to the paired comparison of pre-treatment and post-treatment data. The Wilcoxon rank sum test was used to compare data from patients who had undergone splenectomy (splenectomy group) and those who had not (non-splenectomy group). Correlation between variables was performed using Spearman's rank correlation. PASW statistics 18 (IBM SPSS Inc.) was used to analyse the data. A p value of <0.05 was considered statistically significant.

Results

Patient characteristics

Thirty patients with β-thal/HbE were initially enrolled in the study, and 27 (90%) patients completed the 12-week deferiprone treatment; two patients were missed the follow-up but continued the treatment until the final visit and one patient dropped out because of non-compliance. The patient characteristics are presented in Table 1, and serum ferritin and haematological data are presented in Table 2. All participants were transfusion dependent and had been taking deferiprone for 1–17 years (median 5 years). Fourteen patients had undergone splenectomy 16.5±6.7 years prior to inclusion in the study.

Differences in red blood cell counts (p<0.01), nucleated red blood cell counts (p<0.001), white blood cell counts (p<0.001), and platelet counts (p<0.0001) were observed between patients in the splenectomy group and those in the non-splenectomy group. Notably, the mean white blood cells, platelet count and nucleated red cell count were approximately twofold, threefold and 80-fold higher, respectively, in patients in the splenectomy group than in those in the non-splenectomy group. Serum ferritin and other haematological characteristics were not significantly different between the two groups.

Twelve weeks of treatment with deferiprone at the dose ranging from 20.8 to 94.5 mg/kg/day (median of 56.6 mg/kg/day) did not significantly alter serum ferritin and haematological parameters in either patient group, except for a decrease in red blood cell counts (p=0.021) in the splenectomy group.

Serum concentration of deferiprone

Serum levels of deferiprone at pre-dosing and post-dosing of each visit were measured (Figure 2). The baseline level of deferiprone before the washout period was 5.5 µmol/l. The median of the trough level of deferiprone

Table 1. Characteristics of patients with β-thalassaemia/haemoglobin E included in the study.

	Total (n=30)	Non-splenectomy (n=16)	Splenectomy (n=14)
Men to women ratio (n)	18:12	9:7	9:5
Age (years)	26.5 (18–53)	23.5 (18–49)	27.0 (20–53)
Weight (kg)	48.7 (39.8–63.0)	50.4 (43.2–60.0)	48.0 (39.8–63.0)
Splenectomy duration (years)		NA	16.5 (9–32)
Duration of deferiprone treatment (years)	5 (1–17)	5 (1–10)	5 (2–17)
IU RBC/year (n)	24 (12–32)	24 (12–32)	24 (12–24)
DFP dose (mg/kg/d)	56.5 (20.8–94.5)	59.3 (28.5–94.5)	52.9 (20.8–93.4)

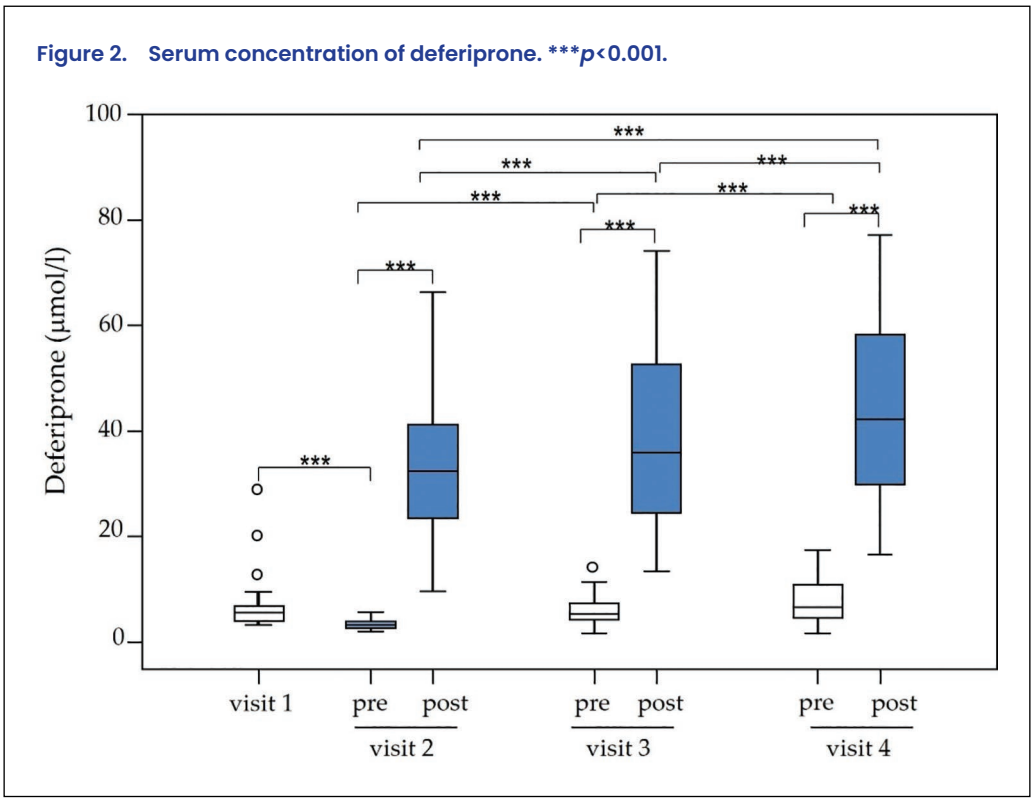
Data are expressed as the median (min–max). DFP, deferiprone; IU RBC, international units of packed red blood cells.

Table 2. Ferritin and haematological data.

	Non-splenectomy		Splenectomy	
	Initial	**12 weeks**	**Initial**	**12 weeks**
Ferritin (ng/ml)	2034 (891–8628)	2316 (627–7767)	1987 (825–11,191)	1923 (746–11,104)
NTBI (μmol/l)	0.8 (0.0–2.4)	2.1 (0–7.7)#	1.8 (1.0–3.0)	2.3 (0.5–5.2)#
Haemoglobin (g/dl)	8.2 (6.4–9.2)	7.8 (6.5–9.2)	7.5 (6.1–8.7)	7.7 (6.4–9.2)
RBC ($\times 10^{12}$/l)	4.0 (2.7–5.2)	3.8 (2.8–5.4)	3.5 (2.7–3.8)*	3.2 (2.6–3.8)*
Nucleated RBC ($\times 10^{9}$/l)	0.3 (0.1–1.7)	0.3 (0.1–1.6)	30.4 (0.7–123.9)*	27.3 (1.5–65.7)*
WBC ($\times 10^{9}$/l)	8.5 (5.4–13.5)	8.4 (4.0–13.9)	14.6 (8.7–22.2)*	12.9 (5.6–19.6)*
Neutrophils ($\times 10^{9}$/l)	4.3 (2.5–10.1)	3.6 (2.2–10.3)	7.3 (4.2–15.0)*	7.4 (0.8–15.2)*
Platelets ($\times 10^{9}$/l)	188 (83–371)	170 (87–346)	593 (295–770)*	635 (281–800)*

Data are expressed as the median (min–max). * $p<0.05$ compared with the respective non-splenectomy group. # $p<0.05$ compared with the respective initial. NTBI, non-transferrin bound iron; RBC, red blood cells; WBC, white blood cells.

Figure 2. Serum concentration of deferiprone. ***$p<0.001$.



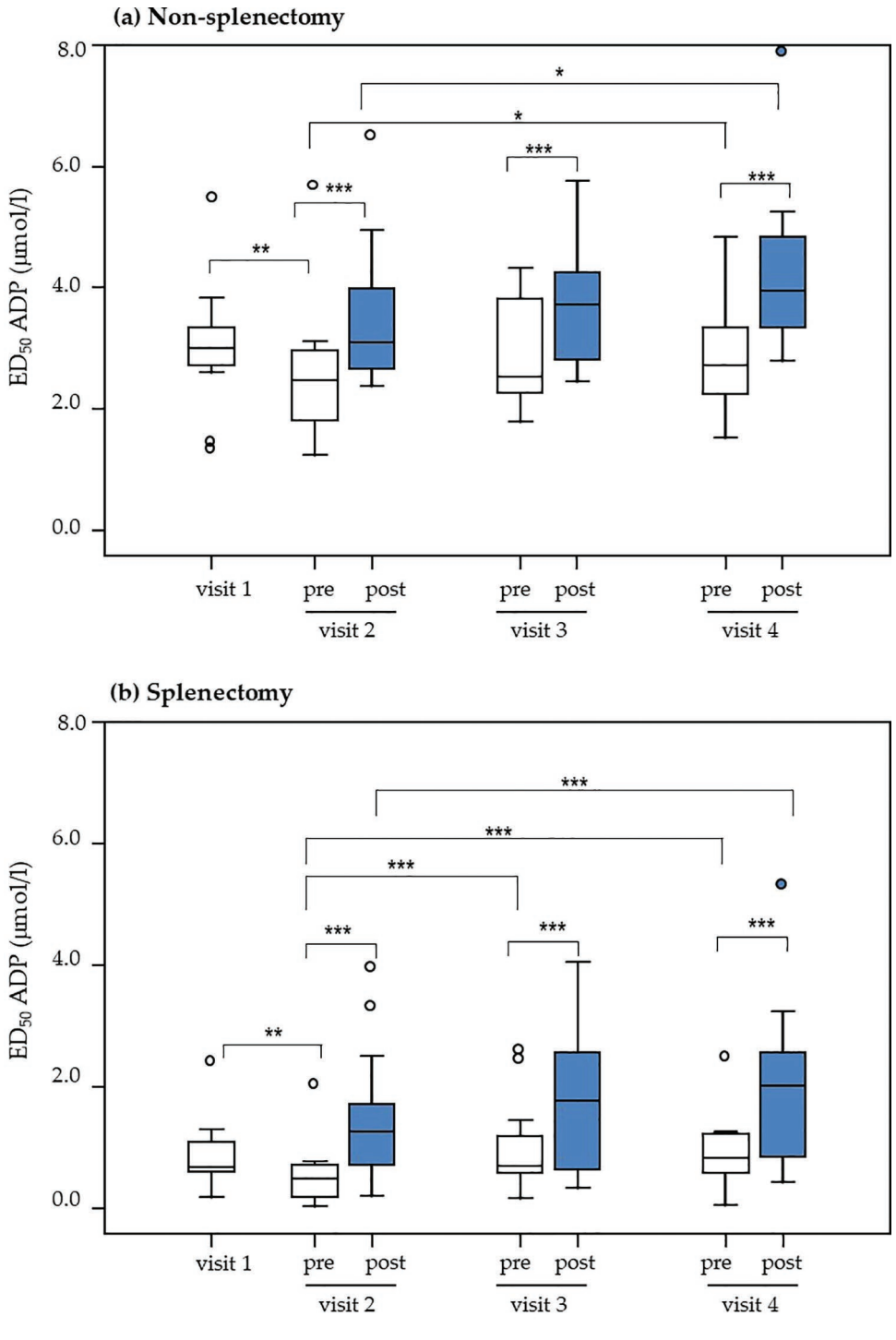
was 3.2 μmol/l at visit 2 and gradually increased over the 12 weeks of treatment to 5.3 and 6.6 μmol/l at visits 3 and 4, respectively.

Moreover, the median of peak level at 2 hours post-dosing was significantly increased from 32.4 μmol/l at visit 2 to 36.0 and 42.3 μmol/l at visits 3 and 4, respectively. However, levels of deferiprone were not significantly different between the two groups of patients.

Effect of deferiprone treatment on platelet aggregation induced by ADP

The response of platelet aggregation induced by ADP was assessed (Figure 3). After a washout period, the median ED_{50} of pre-dosing was 2.48 and 0.48 μmol/l in patients in the splenectomy and non-splenectomy groups, respectively. These values were significantly lower than those at baseline (visit 1).

Figure 3. Deferiprone inhibited ADP-induced platelet aggregation in patients with β-thalassaemia/HbE. ED_{50} value in patients who have not undergone splenectomy (a) and in patients who have undergone splenectomy (b). Visit 1: Baseline, Visit 2: After 4-week washout, Visit 3: After 4 weeks of deferiprone, Visit 4: After 12 weeks of deferiprone. ***p<0.001, **p<0.01, *p<0.05.



The value of ED_{50} of pre-dosing was slightly but significant increased after 12 weeks of deferiprone treatment in both groups of patients. After 2 hours of a single dose of deferiprone, ED_{50} was significantly increased compared with pre-dosing. This change was observed in all visits in both groups of patients. Furthermore, a significantly increased response was observed after 12 weeks of treatment (the median of ED_{50} was 3.1 and 4.0 in visits 2 and 4, respectively, in the non-splenectomy group, and 1.3 and 2.0 in visits 2 and 4, respectively, in the splenectomy group).

Effect of deferiprone treatment on platelet aggregation induced by AA

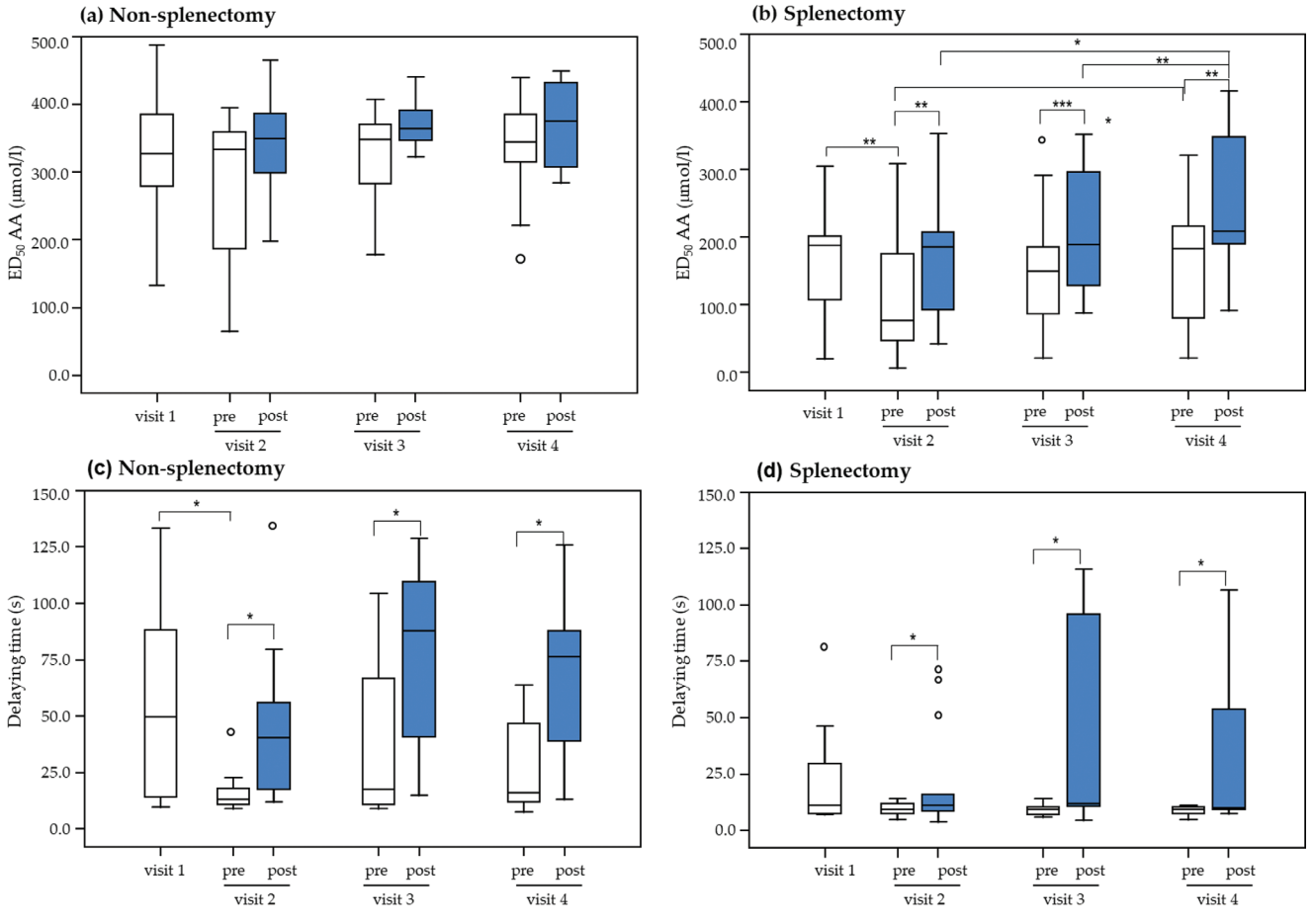
The response of platelet aggregation induced by AA (Figure 4) showed no significant difference in the values of ED_{50} among sampling time points in the non-splenectomy group (Figure 4a). However, an increased response of platelets with AA was observed in patients in the splenectomy group (Figure 4b). The values of ED_{50} of post-dosing significantly increased compared with pre-dosing at all visits ($p<0.005$) and gradually increased; at the end of the study (visit 4), the values of both pre-dosing and post-dosing were significantly increased compared with the initial treatment (visit 2).

Although there was no significant alteration in the values of ED_{50} of AA from patients in the non-splenectomy group, deferiprone significantly prolonged the delay time of platelet aggregation induced by AA in both groups (Figure 4c,d). Delay time was extended from 13.1 to 40.5 seconds after a single dose and to 76.4 seconds at the end of 12-weeks of deferiprone treatment in patients in the non-splenectomy group ($p<0.05$). In the splenectomy group, delay time increased from 7.2 to 10.3 seconds after a single dose and to 10.0 seconds at the end of the 12-week period ($p<0.05$).

Correlation of deferiprone, NTBI and platelet activity

Correlation analysis demonstrated a slight but significant correlation between deferiprone concentration and ED_{50} values for both ADP and AA to induce platelet aggregation (Figure 5a,b). Deferiprone treatment transiently increased NTBI (Figure 5c). However, there was no correlation between NTBI nor molar ratio of deferiprone to NTBI with any parameter of platelet activity.

Figure 4. Deferiprone inhibited arachidonic acid (AA)-induced platelet aggregation in patients with β-thalassaemia/HbE. ED_{50} value in patients who have not undergone splenectomy (a) and in patients who have undergone splenectomy (b); delay time in non-splenectomy (c) and in splenectomy patients (d). Delay time was determined with AA 500 mmol/l. Visit 1: Baseline, Visit 2: After 4-week washout, Visit 3: After 4 weeks of deferiprone, Visit 4: After 12 weeks of deferiprone. ***$p<0.001$, **$p<0.01$, *$p<0.05$.

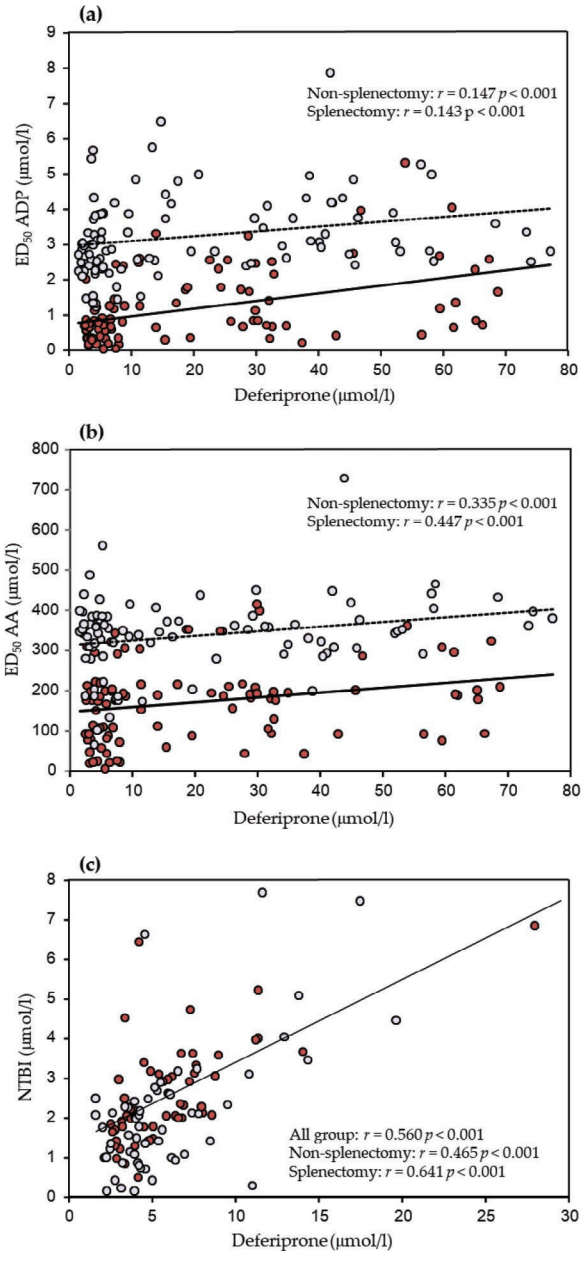


Effect of deferiprone on sP-selectin expression

Levels of sP-selectin were approximately twofold higher in patients in the splenectomy group than in those in the non-splenectomy group (Figure 6).

After a washout period (visit 2), a significant increase in sP-selectin expression was observed in both groups.

Figure 5. Correlation analysis between serum concentration of deferiprone and (a) median effective dose (ED_{50}) value of ADP, (b) ED_{50} value of arachidonic acid (AA) and (c) non-transferrin-bound iron (NTBI) in patients who have not undergone splenectomy (o) and patients who have undergone splenectomy (●).



Interestingly, the significant decrease in sP-selectin levels was observed at 2 hours after taking a single dose of deferiprone in all visits (Figure 6a,b). It should be noted that the suppression by deferiprone was a transient effect: there were no significant differences between 4 and 12 weeks of treatment.

Correlation analysis demonstrated that expression of sP-selectin partly involved in the response of ADP- and AA-induced platelet aggregation, especially in patients in the splenectomy group (Figure 6c,d). The negative correlation between level of sP-selectin and the values of ED_{50} of ADP and AA was r=−0.289 (p<0.01) and r=−0.358 (p<0.001), respectively.

Effect of deferiprone on clotting time

To further assess the effect of deferiprone on secondary haemostasis, aPTT and PT assays were conducted. The values of clotting time in both assays were not different in patients in either group at every visit. In addition, deferiprone treatment did not affect clotting time (Table 3).

Discussion

Platelet hyperactivity has long been recognized and associated to the high risk of thrombosis and vascular complications in β-thal. Venous thromboembolic events are common in deep veins and the incidence rate of thromboembolism in β-thal is 0.9–29%.[19,20] In addition, pulmonary hypertension caused by chronic microembolization in the lung has been found with high incidence in patients who have undergone splenectomy.[21]

There are several factors that contribute to platelet hyperactivity, including increased platelet counts,[22,23] the release of hemin from haemolyzed red blood cells,[24] and oxidative stress inducing the generation of microparticles from red blood cells and platelets.[25,26] Microparticles promote platelet–platelet and platelet–leukocyte aggregation.[27] Our study demonstrated that platelet numbers in patients who have undergone splenectomy were fourfold higher than in patients who had not undergone splenectomy. However, treatment of deferiprone for 12 weeks did not reduce the number of platelets.

With the marked increase in the number of platelets, white blood cells, and nucleated red cells, patients in the splenectomy group had greater sensitivity to both ADP and AA compared with patients in the non-splenectomy group. In addition, the level of sP-selectin, a marker of platelet activation, was significantly higher in patients in the splenectomy group than in patients in the non-splenectomy group. This finding is in line with previous studies that reported the hyperactivation of thalassaemic platelets after splenectomy.[28–31] The

Figure 6. Deferiprone suppresses soluble P-selectin (sP-selectin) levels in patients with β-thalassemia/HbE. sP-selectin in patients who have not undergone splenectomy (a) and in patients who have undergone splenectomy (b); Correlation between sP-selectin levels and median effective dose (ED_{50}) value of ADP (c) and arachidonic acid (AA) (d) in patients who have not undergone splenectomy (o) and patients who have undergone splenectomy (●). Visit 1: Baseline, Visit 2: After 4-week washout, Visit 3: After 4 weeks of deferiprone, Visit 4: After 12 weeks of deferiprone ***p<0.001, **p<0.01, *p<0.05.

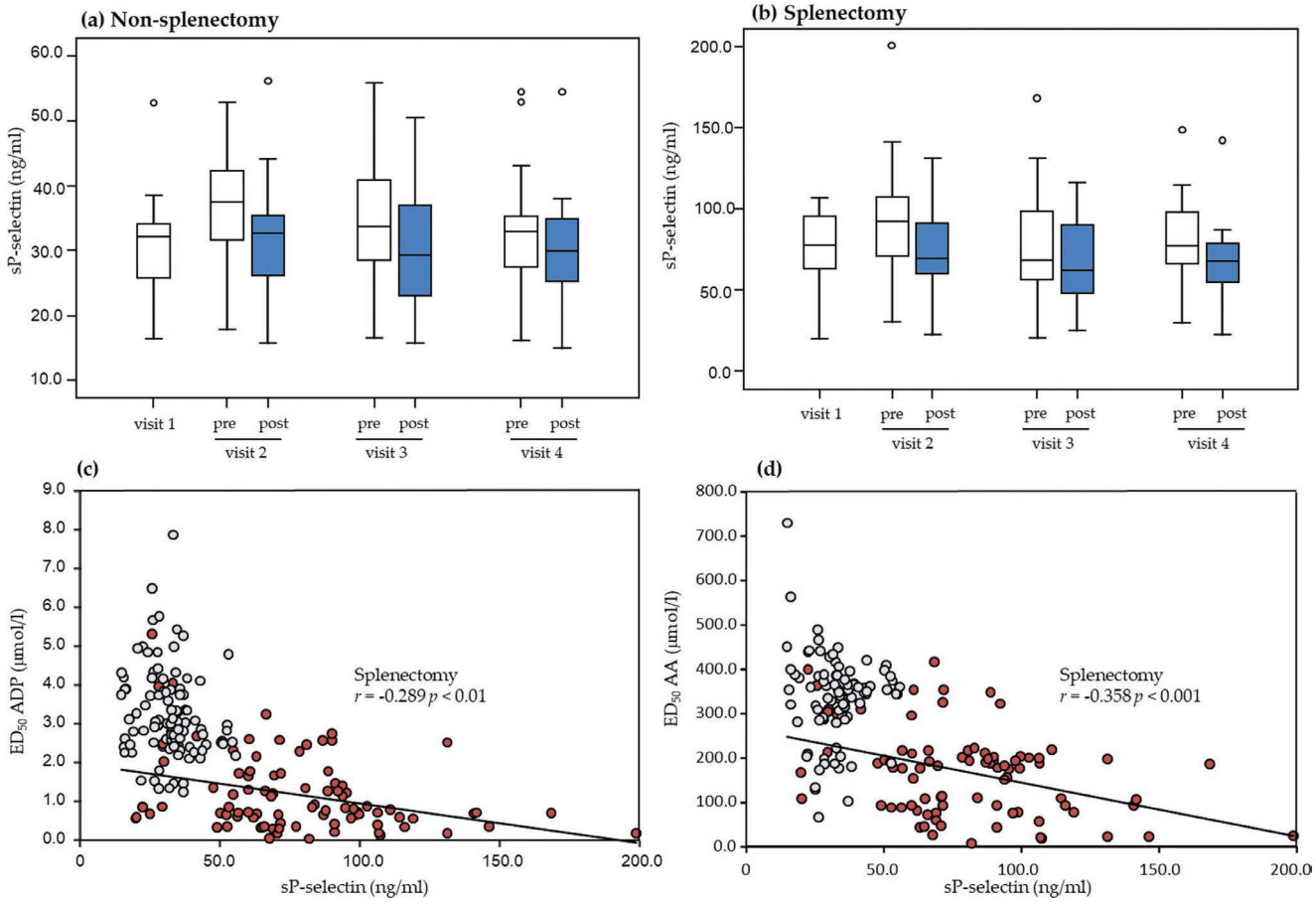


Table 3. Anticoagulant activities in β-thalassaemia/haemoglobin E.

	Non-splenectomy		Splenectomy	
	aPTT (seconds)	**PT (seconds)**	**aPTT (seconds)**	**PT (seconds)**
Before washout (visit 1)	28.6 (25.3–31.7)	13.6 (12.4–14.9)	27.3 (22.8–35.1)	13.2 (12.0–14.7)
Initial (visit 2)	27.5 (22.8–34.2)	13.1 (12.1–15.1)	27.0 (22.0–35.1)	13.0 (12.1–14.7)
4 weeks after deferiprone (visit 3)	29.8 (22.7–37.2)	13.8 (11.8–14.9)	28.3 (23.6–37.0)	13.3 (12.6–14.0)
12 weeks after deferiprone (visit 4)	28.9 (24.0–37.2)	13.2 (12.4–16.1)	27.9 (24.5–38.1)	13.1 (12.2–17.4)

Data are expressed as the median (min–max).
aPTT, activated partial thromboplastin time; PT, prothrombin time.

high level of serum ferritin and high platelet sensitivity indicated that iron overload and hyperactivation of platelets were still present in patients with β-thal/HbE despite chronic iron-chelating treatment and blood transfusion.

The effects of deferiprone were observed in both short-term and long-term treatment. Deferiprone significantly suppressed platelet hyperactivation after 2 hours of a single dose, indicating the direct effect of deferiprone on platelet function of patients with β-thal/HbE, which

may be partly explained by COX1 inhibition. The previous molecular docking study showed that AA had a lower binding energy to COX1 than deferiprone (-8.8 *versus* -5.9 kcal/mol), indicating a higher affinity binding to COX1 enzyme.[13] However, an in vitro study demonstrated that deferiprone dose-dependently inhibited platelet aggregation induced by AA.[13] It could be concluded that deferiprone is reversibly bound to COX1 and competes with AA to inhibit platelet responses in patients with β-thal/HbE.

The ADP-induced platelet aggregation involves mainly two receptors, P_2Y_1 and P_2Y_{12}. P_2Y_1 couples to $G_{q\alpha}$, resulting in granule secretion and TXA_2 formation by inhibition of the COX1 enzyme. The P_2Y_{12} receptor couples to $G_{i\alpha}$ leading to activation of GPIIb/IIIa receptors *via* the cAMP pathway.[32] The in vitro results showed that deferiprone did not affect cAMP levels at lower concentrations of 4 mmol/l, while it inhibited COX1 activity at ED_{50} of 0.33 mmol/l. This implied that deferiprone at serum concentrations did not act on the ADP–cAMP pathway, and the antiplatelet platelet activity of deferiprone may occur mainly through COX1 inhibition. However, the binding affinities of deferiprone to platelet surface receptors require studies to clarify its mechanism of direct antiplatelet activity.

Our data also suggested that long-term treatment with deferiprone may have a protective effect on platelet hyperactivity in patients with β-thal/HbE. Platelets were more sensitive to both agonists (AA and ADP) after 4 weeks of no deferiprone (visit 2). However, the responses returned to baseline after 4 weeks of deferiprone treatment. Therefore, long-term treatment is necessary to maintain the protective effects of deferiprone.

sP-selectin is a reliable marker of platelet activation.[33] The increase in plasma sP-selectin level is closely correlated to the outcomes of acute coronary syndromes both in patients with atherosclerosis and in patients with atherosclerotic risk factors.[34] The significant decrease in sP-selectin levels after deferiprone treatment may be beneficial in preventing thrombotic events in patients with β-thal/HbE regardless of splenectomy status. sP-selectin is shed from the activated platelet membrane. The expression of P-selectin enhances platelet–leukocyte aggregation, resulting in the upregulation of tissue factors and several cytokines in leukocytes.[35] This contributes to the function of platelets on the coagulation pathways and inflammatory processes.[36–38] While iron overload and haemolyzed red cells promote an inflammatory response in patients with thalassaemia,[39] the decrease in sP-selectin levels after treatment with deferiprone may also contribute to improving inflammatory responses in patients with β-thal/HbE.

Iron overload in thalassaemia generates reactive oxygen species (ROS), including $O_2{\bullet}^-$, H_2O_2 and •OH *via* Haber–Weiss and Fenton pathways[40] and promote lipid peroxidation,[41] leading to atherothrombosis complications. Deferiprone also has antioxidant activity and can prevent foam cell formation in macrophage cell culture under iron overload.[42] Vitamin E, a chain-breaking antioxidant, has been shown to reduce platelet hyperactivity but does not ameliorate iron overload.[26] Long-term treatment of antioxidant (vitamin E and N-acetylcysteine) cocktails with deferiprone can improve oxidative stress, iron overload, and hypercoagulable state in β-thalassaemia/HbE.[43] Chronic oxidative stress in thalassaemia platelets was evidenced by a higher ROS level than in normal platelets.[44] Interestingly, catalase and H_2O_2 scavengers can inhibit platelet aggregation and ATP release induced by collagen, ADP, and thrombin.[5] Therefore, iron-chelation therapy could theoretically reduce platelet responses via a decrease in oxidative stress.

The roles of iron-induced oxidative stress in platelet responses are supported by the difference in sensitivity of thalassaemia platelets to ADP and AA. Deferiprone decreased thalassaemic platelet sensitivity to ADP more potently than to AA; this phenomenon was not observed in normal platelets. The increase in AA metabolism due to oxidative stress may explain this discrepancy. ROS can stimulate AA release, resulting in platelet activation.[45,46] Therefore, the potentiation of AA metabolism by iron-induced ROS could be involved in the decreased effectiveness of deferiprone in AA-induced platelet aggregation in patients with β-thal/HbE as compared with normal platelets.

Our previous studies using the enhanced permeability and retention spin trapping technique have demonstrated that the increased iron (as measured as NTBI) during deferiprone chelation therapy was not in the form of catalytic iron, particularly if the molar ratio of deferiprone to iron is greater than 3 to 1.[47] NTBI in the chelated form with deferiprone cannot produce hydroxyl radicals when it is fully coordinated in the deferiprone–iron complex. In addition, we did not detect catalytic iron in the long-term treatment of deferiprone.[48] Our present data (Figure 5c) show that the molar ratio of deferiprone to iron was greater than 3 to 1 in almost all the data points. Therefore, we postulate that this form of iron does not contribute to increased oxidative stress.

Conclusion

Chelation therapy with deferiprone alleviated platelet hyperactivity in patients with β-thal/HbE. The effect may result from direct inhibition of COX1 activity and indirectly

via a decrease in oxidative stress. It should be noted that deferiprone at the dose of iron chelation did not modify coagulation times in patients with β-thal/HbE. The short-term inhibitory effects of deferiprone on platelet reactivity may help prevent thrombotic events in patients with β-thal/HbE with a high risk of cardiopulmonary complications. A study limitation is that the platelet function test is merely a surrogate marker of clinical outcomes. Further investigations with a longer follow-up period to observe the incidence of pulmonary hypertension and/or thrombosis in a large number of patients with β-thal/HbE should be performed.

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