Introduction

Thalassemias are a heterogeneous group of inherited anemias resulting from a reduced or an absent synthesis of alpha- or beta-globin chains of hemoglobin. Thalassemia constitutes a major problem in the countries around the Mediterranean Sea, the Middle East and Trans–Caucasus, India, and the Far East.1 The prevalence of thalassemia is high in Thai population. Approximately 20% to 30% of Thai population carry alpha-globin gene abnormality and 10–53% carry beta-globin gene abnormality.2 In patients with transfusion-dependent thalassemia (TDT), red blood cell transfusion has been the mainstay in the management that supports normal growth and suppression of ineffective erythropoiesis. However, regular blood transfusion can cause iron overload that leads to organ damage.3 Excess iron is extremely toxic to all cells of the body and can cause serious and irreversible organ damage, such as cirrhosis, diabetes, heart disease, and hypogonadism.4

There are evidences that TDT patients, especially in beta-thalassemia diseases, have increased oxygen–free radicals and peroxidative cell injury. Several studies demonstrate that iron toxicity especially toxic form such as non-transferrin-bound iron (NTBI) is the major cause of increased reactive oxygen species (ROS). Iron is known to be a catalyst in the formation of ROS, particularly the hydroxyl radical (OH) serving as a Fenton reagent.4

Other Fenton reagents are hemichromes, which are a family of denatured ferric proteins starting from methemoglobin until the complete dissociation of heme from globin particularly at the cell membrane,5 damaging vital organs as well as the hematopoietic system. Cellular damage associated with iron overload has been attributed to the increased production of ROS which exceeds cellular defense capacities. Reactive oxygen species are capable of causing oxidative damage to macromolecules, leading to lipid peroxidation, oxidation of amino acid side chains, and oxidation of polypeptide chains and finally leading to cell death.4,5 Depletion of lipid-soluble antioxidants has been reported in beta-thalassemia major.6

Infections are major complications and the second most common cause of mortality in thalassemia. Predisposing factors for infections in thalassemic patients include severe anemia, iron overload, splenectomy, and immune abnormalities including low WBC and impair WBC function.7,8

In this study, we aimed to investigate the viability of normal peripheral blood mononuclear cells (PBMCs) when incubated with TDT patient’s serum in comparison with serum incubated with TDT patient’s serum in comparison with control sera. PBMCs viability was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay at 24, 48, and 72 hours. Sixty-nine TDT patients and 22 healthy controls were enrolled. The mean of PBMCs viability after incubation with serum from TDT patients was lower than that with the controls (88.65% vs 103.56% at 24 hours, 78.77% vs 112.04% at 48 hours, and 71.18% vs 132.16% at 72 hours, respectively). High serum ferritin level (correlation −0.29, P < .05) and white blood cell (WBC) count negatively affected cell viability (correlation −2.86, P = .05). From multivariate analysis, serum ferritin level is the only significant risk factor that is independently associated with cell viability (correlation −11.42, P < .001). Our findings showed that TDT patient’s serum causes decreased cell viability. Serum ferritin level was a significant independent factor influencing cell viability.
from normal controls and to evaluate the clinical factors that affected normal PBMCs viability.

**Methods**

**Patients**

Our study is a cross-sectional study that was carried out at the Cell Culture and Molecular Lab at Department of Biochemistry of Chiang Mai University. The patients with TDT were recruited from the Hematology Division, Department of Internal Medicine, Chiang Mai University, Chiang Mai, Thailand.

**Subjects and clinical samples**

The TDT patients at the age above 18 years old were enrolled in this study. Patients who were known to have viral hepatitis and HIV infection were excluded. All patients have been previously molecularly diagnosed for type of thalassemia including beta-thalassemia major and beta-thalassemia/hemoglobin E. Patients received history taking and physical examination and provided written informed consent. This study was approved by the ethical research committee, Faculty of Medicine, Chiang Mai University.

Blood samples were collected from a peripheral vein into vacutainers containing ethylenediaminetetraacetic acid (EDTA) and heparinized tubes. The samples were collected from thalassemia patients at the duration of at least 4 weeks after the previous transfusion. All thalassemia patients were evaluated for complete blood count, liver function test (ie, aspartate aminotransferase and alanine aminotransferase), ferritin, NTBI, blood urine nitrogen, serum creatinine, and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assays, and underwent an MRI scan to evaluate cardiac T2* and liver iron concentration (LIC).

Blood donor volunteers, age 20 to 35 years old, were enrolled as controls. All gave informed consent. Blood samples were collected before blood donation.

**PBMCs isolation**

Peripheral blood mononuclear cells were isolated from theuffy coat part of whole blood from the Blood Donation Center, Maha Raj Nakorn Chiang Mai Hospital, Chiang Mai, Thailand. The cells inuffy coat were separated by Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA) following density gradient centrifugation standard protocols. Briefly, the whole blood with buffy coat was diluted with 1× volume of phosphate-buffered saline (PBS, pH 7.2). Twenty milliliter of diluted blood cells were carefully overlaid over 10 mL of Ficoll-Hypaque, then centrifuged at 400 × g for 30 minutes at room temperature. The upper layer was aspirated leaving the mononuclear cell layer undisturbed at the interphase and then carefully removed the mononuclear cell layer to a new tube. The PBMCs were washed twice by using PBS and centrifuged at 300 × g for 10 minutes at room temperature and the precipitated PBMCs were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 Units/mL penicillin, and 100 µg/mL streptomycin at 37°C in a 5% CO2 atmosphere. The PBMCs were counted and plated on 96-well-plates at 1 × 105 cells per well with 100 µL of incomplete RPMI media.

**MTT tetrazolium assays**

MTT assay (MTT tetrazolium assays) is a widely used cell-based assay to determine cell proliferation. The direct cytotoxic effect and referred cell death can be obtained from the experiment. The principle of this assay is that MTT can readily penetrate viable eukaryotic cells with active metabolism to convert MTT dye to a purple-colored formazan product with an absorbance maximum near 570 nm. However, dead cells lose the mitochondrial enzymatic activity to convert MTT dye to be formazan (Figure 1). In this study, the PBMCs were treated with 100 µL of RPMI plus 10% patient serum, or 10% FBS as cell culture control, or 10% normal human serum. Patients’ serum samples, FBS, and normal human serum samples were all inactivated by incubating at 56°C for 30 minutes. The treated PBMCs were incubated for 24, 48, and 72 hours and then 15 µL of MTT reagent were added and incubated for 4 hours. After that the media were removed and 100 µL of dimethyl sulfoxide was added to dissolve the formazan crystal (purple precipitate). The absorbance at 540 nm with reference to wave length of 630 nm was measured by a microplate reader (Biotek, Winooski, VT, USA). Percent cell viability was calculated by comparison with the FBS-treated PBMCs as 100% cell viability.

The PBMCs were treated with thalassemia serum and normal volunteer serum to compare the inhibitory effect on cell growth or proliferation. There are 3 conditions of serum samples, which are thalassemia patients’ serum, normal human serum, and FBS. The normal human serum is the serum from the healthy volunteer who is not a thalassemic or anemic patient. Fetal bovine serum was used in an in-vitro culture system to maintain the cell viability and used as a negative control before employing thalassemic and normal human.

**Statistical analysis**

Primary end point was to evaluate the factors that affect PBMCs viability after incubated with serum from TDT patients. The secondary end point is to compare PBMCs viability when incubated with serum from TDT patients or controls. Patients’ characteristics of the study population were summarized using descriptive statistical methods. The data were expressed as mean ± SD or median (minimum to maximum). Pearson correlation was used to find the association.
between factor and PBMCs viability. The difference of PBMCs viability in 3 time points (24, 48, and 72 hours) was evaluated by Generalized Estimating Equation and Bonferroni method for multiple comparisons. The statistical significance level was set at $P<.05$.

**Results**

From December 1, 2015, to August 25, 2016, 69 TDT patients and 22 healthy controls were enrolled. The median age of patients was 26 years (range, 18-57) and the median age of normal volunteer was 26 years (range, 20-35). Forty-two patients (60.87%) were female. Most of the patients had beta-thalassemia/hemoglobin E (41 patients, 59.42%) and most were splenectomized (49 patients, 71.01%). Mean pre-transfusion hemoglobin (Hb) was 7.08 ± 1.07 g/dL. Median ferritin level was 1673 ng/mL (range, 274-6079) and 44.93% of patients had ferritin level between 1000 and 2500 ng/mL. All patients received iron chelators. Forty-nine patients (71.01%) received single agents iron chelators (desferrioxamine [DFO] 16 patients [23.19%], deferiprone [DFP] 28 patients [40.58%], and deferasirox [DFX] 5 patients [7.25%]). Twenty patients received combination of iron chelators (DFO + DFP 16 patients [23.19%], DFO + DFX 4 patients [5.80%]). Median LIC was 14.35 mg Fe/g dry weight (range, 1.3-27), Median cardiac T2* was 38.4 ms (range, 3.7-75.4). Patient characteristics are shown in Table 1. There was no significant effects of the following factors on PBMCs viability: age, type of thalassemia, splenectomy, NTBI, cardiac T2*, type of iron chelators, liver enzymes, and kidney function (Table 1).

**Reduction of PBMCs viability when treated with serum from TDT patients in comparison with controls**

Peripheral blood mononuclear cells viability after treatment with serum from TDT patients was significantly lower than that of controls in a time-dependent manner (Figure 2).

**Factors affecting PBMCs viability after incubation with serum from TDT patients**

In univariate analysis, the increase in ferritin level (>1000, 1000-2500, >2500) had a significant negative effect on cell viability and had a correlation with incubation time, at 48 and 72 hours ($P<.001$). The cell viability at 48 and 72 hours decreased when compared with that of 24 hours (Table 2).

Another factor which affected PBMCs viability was WBC number ($P=.05$). The increase of WBC count had negative effect on cell viability (correlation $-2.86$ at 24 hours, $-0.07$ at 48 hours, $-0.12$ at 72 hours, $P=.05$) (Table 3).

The LIC had a trend to influence on PBMCs cell viability by LIC $>7$ mg/g, which had a decreased cell viability when compared in the range of LIC $\leq 7$ mg Fe/g dry weight (mean percent cell viability at 24 hours, 87.03% vs 97.23%; and at 72 hours, 76.72% vs 89.48%, $P=.0621$, respectively) (Table 2).

By using statistics of multivariate analysis, which included 3 factors: 3 ranges of serum ferritin (>1000, 1000-2500, >2500), WBC count, and LIC, it was found that only serum ferritin had a significantly negative effect on PBMC viability (correlation $-14.10$ in ferritin group of 1000-2500 ng/mL, $-19.45$ in group $>2500$ ng/mL; $P\leq .01$, respectively) (Table 4).

**Discussion**

In this study, MTT reduction assays were used to evaluate cell viability. Transfusion-dependent thalassemia patients’ serum reduced PBMC viability more than the age-matched controls, reflecting that TDTs’ serum was more cytotoxic.

Fibach et al$^{10}$ reports that the depressed proliferative response of thalassemic PBMCs is due to the decreased level of intracellular glutathione (GSH) in PBMCs of beta-thalassemia major patients when compared with healthy controls. Several reports have shown that thalassemic patients have increased oxidative stress and reduced cellular enzymatic antioxidants and antioxidant molecules, especially GSH. Thus, GSH plays...
the pivotal roles of intracellular antioxidants. The study also supports the previous findings by demonstrating the higher cellular damage effect of serum from TDT patients. As the effect is correlated positively with serum ferritin level, it is likely initiated via iron-related oxidative injury and leads to cell death.

Oxidative stress exerts a critical role in the pathological process of several chronic diseases, such as diabetes, neurodegenerative diseases, cancers, and also thalassemia. High serum malondialdehyde and serum ferritin levels in patients with thalassemia were significant risk factors for renal cell damage in such patients.11 There is a recent report of the role of metal, such as manganese, in the pathogenesis of neurodegenerative diseases.12 The redox homeostasis is important in controlling oxidative stress related cell death, such as thioredoxin, an antioxidant system, that controls cell fate.13

Another mechanism of iron-related damage is an injury to the cell membrane. The injury can affect the cytoskeletal membrane protein network, such as band 4.1. Band 4.1 partially loses its thiol group with a decreased ability to participate in formation of the spectrin-actin-band 4.1 ternary complex or spectrin plus band 4.1 cross-linked actin.14 Therefore, these abnormal cells are removed by the macrophages to the reticuloendothelial system.

Ozdemir et al15 reported the antioxidant supplementation with oral N-acetylcysteine (NAC) and vitamin E in children with beta-thalassemia. It was found that with antioxidant supplementation, there was a reduction in oxidative stress status and an increased pre-transfusion Hb levels. N-acetylcysteine decreases mononuclear DNA damage. Hence, antioxidants may have a role to reduce cell death in the present model of PBMCs treated with TDT patients’ serum samples but it needs further investigation to confirm this hypothesis such as determination of the effect of pre-treatment with NAC or other antioxidants. In addition, total oxidative status index (OSI) and

| Table 1. Patient characteristics, and clinical and laboratory data of TDT patients. |
|-----------------------------------------------|
| **PATIENT**                                   |
| **(N = 69)**                                  |
| Median age (years)                            | 26 (18-57) |
| Sex (%)                                       |            |
| Men                                           | 27 (39.13%)|
| Women                                         | 42 (60.87%)|
| BMI (kg/m²)                                   | 19.27 ± 1.95|
| Types of thalassemia, n (%)                   |            |
| Beta-thalassemia major                        | 26 (37.68%)|
| Beta-thalassemia hemoglobin E                 | 41 (59.42%)|
| Other                                         | 2 (2.90%)  |
| Splenectomy, n (%)                            | 49 (71.01%)|
| Hemoglobin pre-transfusion (g/dL)              | 7.08 ± 1.07|
| Mean white blood cell (cell/mm³)              | 12710 (3570-31100) |
| Mean blood transfusion (unit/mo)              | 1.54 ± 0.49|
| Ferritin (ng/mL)                              |            |
| Median (range)                                | 1673 (273-6079) |
| <1000                                         | 20 (28.99%)|
| 1000-2500                                     | 31 (44.93%)|
| >2500                                         | 18 (26.09%)|
| LIC (mg/g)                                    |            |
| Median (range)                                | 14.35 (1.3-27) |
| <7                                            | 7 (10.94)  |
| 7-15                                          | 28 (43.75) |
| >15                                           | 29 (45.31) |
| Cardiac T2* (ms)                              |            |
| >20                                           | 56 (90.32%)|
| 10-20                                         | 1 (1.61%)  |
| <10                                           | 5 (8.06%)  |
| Iron chelators                                |            |
| Desferrioxamine (DFO)                         | 14 (20.28%)|
| Deferiprone (DFP)                             | 28 (40.57%)|
| Deferasirox (DFX)                             | 5 (7.24%)  |
| DFO + DFP                                     | 16 (23.19%)|
| DFO + DFX                                     | 4 (5.79%)  |
| Kidney function (CrCl) (mL/min/1.73 m2)       |            |
| >90                                           | 59 (89.39%)|
| 60-89                                         | 5 (7.58%)  |
| 30-59                                         | 1 (1.52%)  |
| <30                                           | 1 (1.52%)  |
| ALT mean ± SD                                 | 34.22 ± 27.78|
| AST mean ± SD                                 | 39.59 ± 23.43|

**Abbreviations:** ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CrCl, creatinine clearance estimate by Cockcroft-Gault equation; LIC, liver iron concentration; TDT, transfusion-dependent thalassemia.
DNA damage levels are significantly higher and total antioxidant capacity (TAC) levels are significantly lower in the thalassemic children than in the healthy control. In both NAC and vitamin E supplemented groups, mean TAC and OSI levels are decreased. Total antioxidant capacity and pre-transfusion Hb levels are significantly increased after 3 months of pre-treatment with NAC. In the NAC group, DNA damage score also decreases. N-Acetylcyesteine and vitamin E reduce serum oxidative stress and increase pre-transfusion Hb levels in children with beta-thalassemia.15

A significant reduction of GSH in PBMCs and PBMC proliferation of beta-thalassemia major patients are reported.16 The influences and mechanistic roles of immunity on oxidative stress remain unclear, especially in thalassemic patients’ immune

| CHARACTERISTICS       | CELL VIABILITY (MEAN ± SD), N = 69 | REPEATED MEASURES ANOVA |
|-----------------------|-------------------------------------|-------------------------|
|                       | 24 H                                | 48 H                    | 72 H                    | EFFECT | TIME |
| Sex                   |                                     |                         |                         |        |      |
| Men                   | 91.24 ± 20.13                       | 79.53 ± 15.73           | 72.69 ± 17.48           | P = .4938 | P = .000 |
| Women                 | 86.98 ± 18.31                       | 78.28 ± 16.68           | 70.21 ± 17.48           |        |      |
| Ferritin (ng/mL)      |                                     |                         |                         |        |      |
| <1000                 | 101.45 ± 19.63                      | 90.30 ± 15.56           | 82.28 ± 14.72           | P = .0001* | P = .000 |
| 1000-2500             | 85.50 ± 16.96                       | 76.54 ± 14.32           | 66.18 ± 16.62           |        |      |
| >2500                 | 79.84 ± 14.66                       | 69.77 ± 12.94           | 67.47 ± 16.60           |        |      |
| Splenectomy           |                                     |                         |                         |        |      |
| Splenectomy           | 87.92 ± 20.12                       | 77.78 ± 17.16           | 70.92 ± 18.77           | P = .5870 | P = .000 |
| Non-splenectomy       | 90.43 ± 16.32                       | 81.18 ± 13.70           | 71.83 ± 13.86           |        |      |
| Cardiac T2*           |                                     |                         |                         |        |      |
| >20 ms                | 86.31 ± 17.58                       | 76.99 ± 15.41           | 69.16 ± 16.89           | P = .8935 | P = .000 |
| <10 ms                | 90.05 ± 27.70                       | 75.64 ± 18.63           | 69.60 ± 18.87           |        |      |
| Type of thalassemia   |                                     |                         |                         |        |      |
| Beta-thalassemia major| 92.04 ± 18.65                       | 78.76 ± 16.70           | 70.28 ± 17.17           | P = .8359 | P = .000 |
| Beta-thalassemia hemoglobin E | 86.73 ± 19.46       | 79.19 ± 16.12          | 71.96 ± 17.59           |        |      |
| Other                 | 83.74 ± 15.52                       | 70.27 ± 18.50           | 66.88 ± 27.29           |        |      |
| Iron chelator         |                                     |                         |                         |        |      |
| Desferrioxamine (DFO) | 87.58 ± 18.68                       | 76.22 ± 14.50           | 68.87 ± 15.33           | P = .2067 | P = .000 |
| Deferiprone (DFP)     | 95.59 ± 15.06                       | 81.96 ± 15.26           | 74.65 ± 18.85           |        |      |
| Deferasirox (DFX)     | 91.18 ± 25.44                       | 81.61 ± 16.91           | 76.64 ± 18.08           |        |      |
| DFO + DFP             | 80.79 ± 21.20                       | 76.05 ± 20.29           | 67.99 ± 18.34           |        |      |
| DFO + DFX             | 72.54 ± 12.46                       | 73.93 ± 12.44           | 62.14 ± 3.92            |        |      |
| LIC (mg Fe/g dry weight) |                                     |                         |                         |        |      |
| <7                    | 97.23 ± 20.43                       | 89.48 ± 16.11           | 80.53 ± 14.15           | P = .0621 | P = .000 |
| ⩾7                    | 87.03 ± 18.38                       | 76.72 ± 14.83           | 69.54 ± 17.60           |        |      |

Abbreviations: ANOVA, analysis of variance; LIC, liver iron concentration.

*P < .05 as statistically significant compared with ferritin <1000.
system. The previous study reported that the increase in oxidative stress in thalassemic serum when compared with normal control is due to the reduction of intracellular GSH in thalassemic PBMCs, which also impairs T-cell activation in vitro.17 Lower mean T-cell population is present in thalassemia major but not the trait. The helper/suppressor ratio is decreased in patients with evidence of hypersplenism. Patients who have suffered from pneumonia or hepatitis manifest lower mean T cell count, depletion of helper T-cells and a decrease in helper/suppressor cell ratio. The inhibitory effect delays cutaneous hypersensitivity. Study of the cell-mediated immunity in patients with thalassemia is beneficial to detect those who are more susceptible to infections.17

Furthermore, iron participating in the Fenton reaction with less potent ROS as the substrate produces more a potent secondary ROS. Under pathological conditions, when ROS are generated, iron catalyzes the reaction of superoxide anion radicals (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) generation, resulting in hydroxyl radical (·OH) formation. The hydroxyl radical is the most reactive and the shortest half-life (10$^{-12}$ seconds), which causes severe damage to biological molecules, including lipids, proteins, and nucleic acids.18 Severe cell damage associated with iron overload has been attributed to the emergence of excessive levels of iron, which promotes production of ROS exceeding cellular defense capacities.19 There are many research data reported that iron overload causes significant high oxidative

### Table 3. PBMCs viability after incubation for 24, 48, and 72 hours to demonstrate the correlation with the liver iron concentration.

| VARIABLES                  | PEARSON CORRELATION | GEE METHOD |
|----------------------------|---------------------|------------|
|                            | 24 H | 48 H | 72 H | EFFECT | TIME |
| Age (years)                | 0.0287 | 0.0287 | 0.0075 | P = .805 | P = .113 |
| Weight                     | 0.0068 | −0.0499 | 0.0420 | P = .856 | P = .322 |
| BMI                        | −0.1069 | −0.1883 | −0.1243 | P = .475 | P = .583 |
| LIC                        | −0.2231 | −0.2967 | −0.1467 | P = .095 | P = .005 |
| Rate of blood transfusion  | 0.1320 | 0.1494 | 0.0630 | P = .286 | P = .184 |
| Cardiac T2*                | −0.1177 | −0.0053 | −0.0291 | P = .421 | P = .014 |
| Pre-transfusion Hb         | −0.0149 | −0.0329 | 0.0069 | P = .844 | P = .315 |
| Pre-transfusion Plt        | −0.0721 | −0.0724 | −0.0409 | P = .589 | P = .001 |
| Pre-transfusion WBC        | −0.2866 | −0.0732 | −0.1234 | P = .050 | P = .000 |
| AST                        | −0.2112 | −0.3121 | −0.2631 | P = .201 | P = .004 |
| ALT                        | −0.2398 | −0.2922 | −0.2535 | P = .131 | P = .000 |
| Kidney function (CrCl)     | −0.1238 | −0.0874 | −0.1442 | P = .556 | P = .025 |

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CrCl, creatinine clearance estimate by Cockcroft-Gault equation; GEE, Generalized Estimating Equation; PBMCs, peripheral blood mononuclear cells; pre-transfusion Hb, mean pre-transfusion hemoglobin; pre-transfusion Plt, mean pre-transfusion platelet; WBC, white blood cell.

### Table 4. Multivariate analysis of correlations between clinical factors and PBMCs viability.

| VARIABLES                  | COEFFICIENT | STANDARD ERROR (SE) | 95% CI          | P     |
|----------------------------|-------------|---------------------|-----------------|-------|
| Ferritin (ng/mL)           |             |                     |                 |       |
| <1000                      | 0           |                     |                 |       |
| 1000-2500                  | −14.1062    | 3.2569              | −20.4897 to −7.7227 | .000  |
| >2500                      | −19.4472    | 4.4750              | −28.2181 to −10.6763 | .000  |
| LIC (mg Fe/g dry weight)   | 0.1715      | 0.2647              | −0.3472 to 0.6903 | .517  |
| White blood cell (cell/mm$^3$) | −0.0002   | 0.0002              | −0.0006 to 0.0002 | .313  |
| Time (hours)               | −0.3625     | 0.0573              | −0.4748 to −0.2502 | .000  |

Abbreviations: CI, confidence interval; LIC: liver iron concentration; PBMCs, peripheral blood mononuclear cells.
stress and DNA damage. Nevertheless, from the present study, it is shown that serum ferritin, intracellular iron-binding, and accumulating proteins have high inhibitory effects on PBMC viability.

Thalassemic patients, whose serum ferritin were 1000 to 2500 ng/mL, had lower PBMC viability of 14.10% when compared with that incubated with patients’ serum samples ferritin concentrations below than 1000 ng/mL. This effect was also strikingly seen in the patients, whose ferritin levels were more than 2500 ng/mL, the PBMCs viability was 19.44% when compared with the PBMC group incubated with thalassemic serum ferritin levels below 1000 ng/mL. These results can be explained that higher level of iron overload led to more ROS production via Fenton chemistry and Harber-Weiss reaction, causing more potent oxidative stress and subsequently more cell damage and death. It was shown that ferrous iron is a cofactor of Fenton reaction and Harber-Weiss reactions.20

Result from iron-induced oxidative stress has the same effect in other diseases, for example, hereditary hemochromatosis.21 Iron overload also has the effect on monocyte phagocytosis as shown by imminent oxidative stress, which destabilizes the lysosomes of macrophages, leading to losing their protective roles and undergoing cell death.22 In lung hemochromatosis, the epithelial cells also release metal (Fe) as ferritin and transferrin for clearance by the reticuloendothelial system.23 Iron chelation plays a major role to reduce oxidative stress and prevents organ damages from iron overload but the mechanistic roles of antioxidants in iron overload remains unclear.19 Serum ferritin is a hallmark of inadequate iron chelation and vulnerability to develop iron-overloaded complications.4 There are studies in rats with iron-overloaded by using oral gavage model, the levels of liver enzymes (aspartate aminotransferase and alanine aminotransferase) are investigated in rats, whether they receive iron overload and/or antioxidants. However, the levels of antioxidants in the rats that receive antioxidants tend to have lower levels of transaminases, indicating less hepatocyte injury, but not statistically significant.23

The limitation of the present research was that total oxidative stress or intracellular GSH level is not evaluated. The parameters, which were reported, were the final end result of cell cytotoxicity or cell viability not at the signaling cascade or molecular levels, such as ROS and GSH levels or by using cell death parameters, such as caspase enzyme activity.24 The ferritin levels (total iron-loaded model) have a significant positive correlation with age parameter (P < .001) and serum transferrin receptor level. Biomarkers with oxidant activity are also significantly increased in thalassemic patients compared with controls, for example, malondialdehyde, NTBI, and GSSG (glutathione-disulfide)/GSH ratio; especially in severe thalassemia. The activities of antioxidant enzymes, such as glutathione reductase (GR), glutathione peroxidase (GPx), and superoxide peroxidase (SOD), are significantly reduced in such patients, especially in the severe type.25

Erythrocyte oxidative stress is related to the toxic effect of hemolytic drug treatment or aging or a model of prolonged aerobic condition reintroduced (ischemia-reperfusion injury), especially found in premature childbirth. The released iron plays a pivotal role in membrane protein oxidation and lipid peroxidation, leading to senescent cell antigen (SCA) formation, which is the major pathway for red blood cell removal in reticuloendothelial system. Iron chelators are able to enter cells, such as ferrozine, quercetin, and fluor-benzil-pyridoxal hydrazone, which prevents both membrane protein oxidation and SCA formation. The fetal hemoglobin is more susceptible to release iron than adult hemoglobin. In newborns, the release of iron in erythrocytes is correlated with plasma nonprotein-bound iron. This report indicates that the iron-binding proteins are able to release free iron into the circulation.26

In conclusion, serum from TDT patients inhibits normal PBMCs growth and proliferation, which led to a higher fraction of cell death than normal serum incubated with the normal PBMCs. The most important factor which affects cell viability is serum ferritin. The mechanism(s) of PBMCs’ death needs further investigation. From the previous studies, the causative agents might be due to the agents causing oxidative stress in thalassemic serum or depletion of antioxidants or antioxidant enzymes in the patients. PBMCs may undergo apoptosis, further study such as the activities of caspase-3 in treated PBMCs lysate would be further provided for the evidence of this hypothesis.

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Author Contributions
NP designed the study, collected and analyzed the data, and wrote the manuscript; RB supervised experimental study and give critical comments; AT, corresponding author, conceived and designed the study, obtained research funding, supervised the study, collected and analyzed the data, and wrote the manuscript; JP and PK performed experimental study; KF, TR, CC, ER, LN, and PC gave critical comments and revised and approved the final manuscript. All authors drafted the article, revised it critically for important intellectual content, and approved the final version of the manuscript to be published.

Ethical Approval
This study was approved by the ethical research committee, Faculty of Medicine, Chiang Mai University (Study code: MED-2559-03811).

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