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Original article

Effect of vitamin E supplementation on oxidative stress in non-transfusion-dependent thalassemia

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Summary. Excessive free radical production is found in thalassemic patients, and this can lead to many complications. The objective of this study was to determine the effect of vitamin E supplementation on oxidative stress in the young patients with non-transfusion-dependent thalassemia (NTDT). Seventeen patients aged between 5-20 years participated in this study. They were divided into vitamin E group (supplemented with 10 IU/kg/day of vitamin E) and control group (no vitamin E supplementation). The levels of serum vitamin E and plasma malondialdehyde (MDA), hemolysis and complete blood count (CBC) were evaluated at baseline and at the end of the study (week 12). The results showed that after 12 weeks of the study, the patients in the vitamin E group had significantly increased serum vitamin E levels ($p < 0.01$) and significantly decreased plasma MDA levels ($p < 0.05$) as compared to baseline, and these levels significantly differed from those in the control group ($p < 0.05$). In vitamin E group, the percentage of hemolysis induced by 2, 2'-azobis-2-methyl-propanimidamide dihydrochloride (AAPH) was significantly decreased as compared to baseline ($p < 0.05$) after vitamin E supplementation. There was no change in hemoglobin and hematocrit throughout the study. The results indicated that vitamin E supplementation in NTDT may benefit these patients in alleviating complications from oxidative stress.

Key words: Vitamin E, oxidative stress, non-transfusion-dependent thalassemia, hemolysis

Introduction

Thalassemia is the most common hereditary chronic hemolytic anemia due to defective globin synthesis. Patients with non-transfusion-dependent thalassemia (NTDT) have moderate anemia and require only occasional blood transfusion when having health conditions such as fever and infection. In patients' erythrocytes, free α-hemoglobin chain causes the release of the iron atom from heme and consequently catalyzes the production of reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$). The up-regulation of these ROS could cause the damage to the erythroid precursor and mature erythrocyte membrane leading to clinical hemolysis in thalassemic patients.

Normally, there is a mechanism within the cells that balance the level of oxidant and oxygen radical. Besides the antioxidant machinery involving the activity of antioxidant enzymes, namely superoxide dismutase, catalase and glutathione peroxidase, the antioxidant vitamins obtained from diet were considered

as an important mechanism in regulation of cellular oxidative stress. Vitamin E is a lipid-soluble antioxidant found in vegetable oils, nuts and green leafy vegetables. Alpha-tocopherol is the most active form and powerful biological antioxidant. An important role of vitamin E is to protect the membrane polyunsaturated fatty acids from peroxidation by scavenging peroxyl radicals ($LOO^{\cdot}$) (1). Tocopherol can scavenge ROS by termination of free-radical chain reaction (2). Vitamin E has been shown to effectively reduce $O_2^{\cdot-}$ (3) and $OH^{\cdot}$ (4) production.

Several studies have shown a benefit of vitamin E supplementation in thalassemic patients. In patients with thalassemia intermedia, hypoxemia and pulmonary vascular occlusion were delayed after supplementation of vitamin E (525 IU/d) for 3 months (5). Vitamin E (400 mg/d) has been used in combination with low dose of vitamin C (100 mg/d) to promote antioxidant status and may enhance liver function in thalassemia major children (6). Previuos study also found the reduction in erythrocyte ROS in adult patients with β-thalassemia intermedia who underwent ocassional transfusion after vitamin E supplementation at the dose of 400 IU/d for 3 months (7).

As described above, the patient with thalassemia is associated with oxidative stress due to increased ROS. Previous studies indicated that vitamin E could decrease oxidative stress and lipid peroxidation in adult thalassemia major and intermedia who acquired transfusion dependence. However, the effects of vitamin E supplementation in children with NTDT in the terms of biochemical parameters, lipid peroxidation, and hemolysis remain unclear. Therefore, this study was conducted to investigate these concerns. The information gained from this study may strengthen the knowledge of vitamin E supplementation and benefit the further development of thalassemia therapy.

Methods

Patients

Patients aged 5–20 years who were diagnosed with NTDT by hemoglobin typing from Pediatric Hematology Clinic, Siriraj Hospital were recruited into the study. All of them had hemoglobin levels between 7-9 g/dL and did not require regular transfusional therapy. The patients were not on iron chelation therapy and did not take vitamin E and any antioxidant supplements, e.g. vitamin C and/or curcumin, for at least 3 months prior to the study. They were free from acute or chronic infection or surgical operation for at least 1 month prior to the study. None of them had vitamin E allergy. The study protocol was approved by the Ethics Committee of Faculty of Medicine Siriraj Hospital. The patients received the explanation of experimental protocol, and the written informed consent was obtained before the beginning of the study.

Experimental design

At the beginning of the study (week 0), the patients received nutrition counseling that involved a consideration of energy intake and the proportion of protein, fat, carbohydrate and other nutrients suitable for thalassemia management. Each patient received dietary guideline for thalassemic patients. Additionally, the patients were assigned into a vitamin E group and a control group. The patients in vitamin E group received vitamin E (Mega®, Thailand) 10 IU/kg/day for 12 weeks. The patients in control group did not receive any supplementation. All of them were advised to stay on regular diets as recommended at home and avoid taking other antioxidant supplements such as vitamin C. Each patient was also asked to maintain the level of physical activity throughout the study. At week 0 and at the end of the study (week 12), the weight and height of the patients were measured, and 3-day food intakes were recorded. In addition, peripheral blood sample (10 mL) was obtained from each patient to determine complete blood count (CBC), serum vitamin E levels, plasma malondialdehyde (MDA) levels, and hemolysis. The patients' compliance and adverse effects were assessed by interviewing and counting the remaining capsules of vitamin E at the end of the study.

Dietary assessment

To estimate daily dietary intake, the 3-day food record (2 weekdays and 1 weekend day) was performed by the patients or their families at week 0 and week 12.

The patients were provided with dietary record forms, and were asked to record all items and portions of food consumed including name and method of preparation and cooking. Food portion sizes were estimated using standard household measuring cups and spoons. The food records were calculated for energy, protein, carbohydrate, and fat intakes using the software Thai NutriSurvey version 2.0 (2008) developed for Thai food by Department of Health, Ministry of Public Health and Faculty of Tropical Medicine, Mahidol University, Thailand.

Biochemical assessment

At week 0 and week 12 of the study, peripheral blood samples were collected for determining CBC, serum vitamin E levels, plasma MDA levels, and hemolysis. Serum vitamin E levels were measured by high performance liquid chromatography (HPLC). Plasma MDA levels were determined by the commercial kit (Cayman, Germany) following the manufacturer's protocol.

Determination of hemolysis

Erythrocytes were separated from the plasma by centrifugation at 1,200 rpm (model B708318; Labnet International, Inc, USA) for 5 minutes at room temperature and washed 3 times with phosphate-buffered saline (PBS) pH 7.4. Then, the erythrocytes were diluted to a concentration of 5×10^8 cells/mL in PBS, counted using a hemocytometer. An aliquot of 50 μL of erythrocytes suspension was mixed with ROS generators, H_2O_2 or 2, 2'-azobis-2-methyl-propanimidamide dihydrochloride (AAPH), with different concentrations (absorbance A_{test}) and 50 μL of PBS buffer (pH 7.4) (absorbance A). The mixture was shaken gently while being incubated at 37°C for 2 hours. The absorbance of the supernatant was measured at 570 nm by using spectrophotometer. Triton-X 1% was used as a positive control (absorbance $A_{positive}$). The percentage of hemolysis was calculated by the following equation:

$\%\ \text{Hemolysis} = (A-A_{test})/(A-A_{positive}) \times 100$

A = absorbance of the negative control

A_{test} = absorbance of the sample

$A_{positive}$ = absorbance of the positive control

Statistical analysis

The data were expressed as mean ± SEM. Normal distribution of the variances was tested by Kolmogorov-Smirnov test. Paired *t*-test was used to test for significant difference between the values at week 0 and week 12 within group. Significant difference of values between groups at week 0 was tested by independent *t*-test and at week 12 was tested by analysis of covariance (ANCOVA). Statistical analysis was considered significant at $p < 0.05$.

Results

There were 17 patients in the study (8 males and 9 females). The characteristics of the patients are shown in Table 1. The vitamin E group included 9 patients (2 males and 7 females), and the control group included 8 patients (6 males and 2 females). There was no statistical difference between the patients in two groups regarding age, weight, height, duration of thalassemia, and other vitamin and mineral use.

Effect of vitamin E on dietary intake

Dietary intakes assessed by 3-day food records at week 0 and week 12 in both groups were analyzed (Table 2). The results showed that energy distribution and nutrient intakes of the patients in the vitamin E and control groups at week 0 of the study were not significantly different. It appeared that the patients in both groups had total caloric intake less than the recommendation of the dietary reference intake for Thais 2003. In the vitamin E group, the percentage of energy from protein at week 12 was significantly less than that of the energy at week 0 ($p < 0.05$). At week 12, the amount of carbohydrate consumption in the vitamin E group was significantly greater than that of the consumption at week 0 ($p < 0.01$), and the amount was also significantly greater than that in the control group at week 12 ($p < 0.01$).

Table 1. Characteristics of the patients

Characteristics	Vitamin E group (n = 9) n (%)	Control group (n = 8) n (%)	Total (n = 17) n (%)
Sex			
Male	2 (22.2)	6 (75.0)	8 (47.1)
Female	7 (77.8)	2 (25.0)	9 (52.9)
Age (years)			
5-10	0	1 (12.5)	1 (5.9)
11-15	4 (44.4)	4 (50.0)	8 (47.1)
16-20	5 (55.6)	3 (37.5)	8 (47.1)
Mean of age[1] (years)	15.56 ± 0.73	13.50 ± 1.43	14.59 ± 0.79
Weight[1] (kg)	44.33 ± 1.70	37.80 ± 5.14	41.26 ± 2.62
Height[1] (m)	1.56 ± 2.63	1.48 ± 7.39	1.52 ± 3.73
Duration of thalassemia (years)			
<5	0	1 (12.5)	1 (5.9)
5-10	0	2 (25.0)	2 (11.8)
>10	9 (100.0)	5 (62.5)	14 (82.4)
Mean of duration[1] (years)	13.33 ± 0.78	9.75 ± 1.57	11.65 ± 0.96
Other vitamin and mineral use			
Yes	3 (33.3)	4 (50.0)	7 (41.2)
No	6 (66.7)	4 (50.0)	10 (58.8)

[1]mean ± SEM

Table 2. Energy distribution and nutrient intakes of the patients at baseline and week 12 of the study[1]

Nutrient intake	Vitamin E group (n=9)		Control group (n=8)		Recommended range[2]
	Week 0	Week 12	Week 0	Week 12	
Total energy intake kcal/day	1334.67 ± 149.33	1610.74 ± 98.44	1432.18 ± 126.46	1550.64 ± 144.04	1600 - 1700
Total protein					
kcal/day	246.09 ± 21.68	204.29 ± 11.37†	223.86 ± 16.03	278.66 ± 31.18	
g/day	61.52 ± 5.42	51.07 ± 2.84†	55.96 ± 4.01	69.66 ± 7.79	40 - 41
g/kg/day	1.39 ± 0.12	1.14 ± 0.06*†	1.48 ± 0.11	1.82 ± 0.20	1.2
% of total energy	18.44 ± 1.62	12.68 ± 0.71*†	15.63 ± 1.12	17.97 ± 2.01	
Fat					
kcal/day	405.41 ± 93.05	427.19 ± 72.15	582.26 ± 94.50	562.87 ± 82.74	
g/day	45.05 ± 10.34	47.47 ± 8.02	64.70 ± 10.50	62.54 ± 9.19	-
% of total energy	30.38 ± 6.97	26.52 ± 4.48	40.66 ± 6.60	36.30 ± 5.34	
Carbohydrate					
kcal/day	681.34 ± 75.92	982.38 ± 41.76**‡	628.62 ± 111.34	687.82 ± 88.14	
g/day	170.34 ± 18.98	245.60 ± 10.44**‡	157.16 ± 27.84	171.95 ± 22.03	-
% of total energy	51.05 ± 5.69	60.99 ± 2.59†	43.89 ± 7.77	44.36 ± 5.68	

*[1]mean ± SEM; [2]Recommended ranges obtained from Dietary Reference Intake for Thais 2003, Bureau of Nutrition, Ministry of Public Health; * Significant difference from week 0 within group ($p < 0.05$); ** Significant difference from week 0 within group ($p < 0.01$); [†]Significant difference from the control group at week 12 ($p < 0.05$), [‡]Significant difference from the control group at week 12 ($p < 0.01$)*

Table 3. Laboratory parameters of the patients at week 0 and week 12 of the study[1]

Parameters	Vitamin E group (n=9)		Control group (n=8)	
	Week 0	Week 12	Week 0	Week 12
Complete blood count				
Hb (g/dL)	8.20 ± 0.22	8.06 ± 0.18	7.74 ± 0.20	7.71 ± 0.35
Hct (%)	27.58 ± 0.94	27.07 ± 0.72	26.79 ± 0.67	26.68 ± 1.17
RBC (10^6/mL^3)	4.54 ± 0.27	4.37 ± 0.23	3.87 ± 0.20	3.91 ± 0.22
MCV (fl)	61.68 ± 2.54	62.89 ± 2.88	70.53 ± 4.23	69.94 ± 4.25
MCH (pg)	18.37 ± 0.87	18.68 ± 0.74	20.23 ± 1.02	20.10 ± 0.90
MCHC (g/dL)	29.77 ± 0.64	29.80 ± 0.64	28.79 ± 0.51	28.95 ± 0.69
RDW (%)	30.00 ± 3.71	26.70 ± 0.91	25.24 ± 1.01	25.21 ± 1.09
MPV (fl)	9.63 ± 0.21	9.56 ± 0.22	8.95 ± 0.40	8.99 ± 0.41
Serum vitamin E (μg/dL)	397.44 ± 90.54	1318.24 ± 185.99**‡	393.00 ± 87.42	444.90 ± 80.95
Plasma MDA (μM)	38.11 ± 6.10	28.06 ± 2.94*†	36.94 ± 7.44	63.57 ± 16.70

*[1]mean ± SEM; * Significant difference from baseline within group ($p < 0.05$), ** Significant difference from baseline within group ($p < 0.01$); [†]Significant difference from the control group at week 12 ($p < 0.05$), [‡]Significant difference from the control group at week 12 ($p < 0.01$); Hb = hemoglobin; Hct = hematocrit; RBC = red blood cell; MCV = mean corpuscular volume; MCH= mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; RDW = red cell distribution width; MPV = mean platelet volume; MDA = malondialdehyde*

Effect of vitamin E on CBC, serum vitamin E levels and plasma MDA levels

The results of CBC, serum vitamin E levels and plasma MDA levels are shown in Table 3. It was found that there was no significant difference in serum vitamin E at week 0 between groups; however, at week 12, the serum vitamin E levels were significantly increased ($p < 0.01$) in the vitamin E group. Serum vitamin E levels in the vitamin E group were significantly more than those in the control group ($p < 0.01$). At week 0 of the study, there was no significant difference in plasma MDA levels of the patients in both groups, but after 12-week of vitamin E supplementation, there was a decrease in plasma MDA levels ($p < 0.05$). At week 12, Plasma MDA levels in the vitamin E group were significantly less than those in the control group ($p < 0.05$).

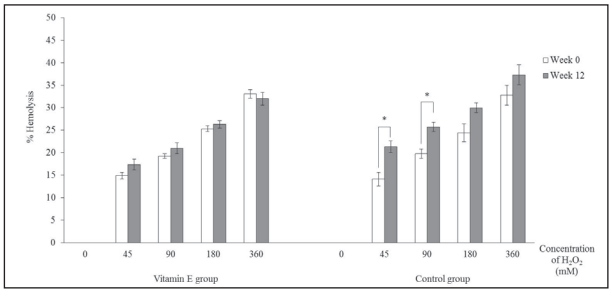

Figure 1. Percentage of hemolysis induced by H_2O_2
* Significant difference from baseline within group ($p < 0.05$)

Effect of vitamin E on hemolysis

Hemolysis generated by H_2O_2

The hemolysis result of erythrocytes treated with H_2O_2 is presented in Figure 1. At week 0 and week 12 of the study, the percentage of hemolysis progressively increased in a dose-dependent manner in both groups. There was no significant difference between the vitamin E group and the control group in the percentage of hemolysis at both time points of the study. After vitamin E supplementation for 12 weeks, the percentage of hemolysis did not significantly differ from baseline. However, at week 12, the percentage of hemolysis in the control group was significantly greater than that of the hemlysis at week 0 with 45 mM H_2O_2 ($p < 0.05$) and 90 mM H_2O_2 ($p < 0.05$).

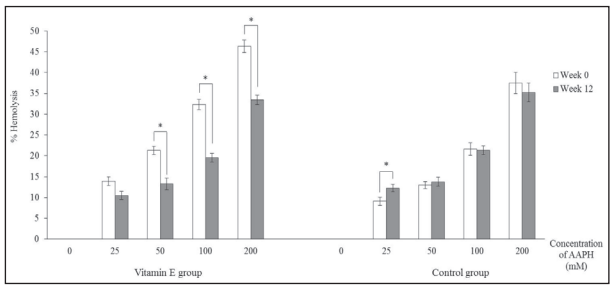

Figure 2. Percentage of hemolysis induced by AAPH
* Significant difference from baseline within group ($p < 0.05$)

Hemolysis generated by AAPH

The influence of vitamin E on hemolysis in the presence of AAPH is shown in Figure 2. At week 0 and week 12 of the study, the percentage of hemolysis progressively increased in a dose-dependent manner in both groups. There was no significant difference between the vitamin E group and the control group in the percentage of hemolysis at both time points of the study. At week 12, the percentage of hemolysis significantly decreased as compared to that of the hemolysis at week 0 with 50 mM AAPH ($p < 0.05$), 100 mM AAPH ($p < 0.05$) and 200 mM AAPH ($p < 0.05$) in the vitamin E group. However, the percentage of hemolysis in the control group at week 12 significantly increased as compared to that of hemolysis at week 0 with 25 mM AAPH ($p < 0.05$).

Discussion

It was well recognized that oxidative stress in patients with thalassemia plays a key role in the progression of the diasease and potentiates the complications including hemolysis. Antioxidant nutrient in food is a valuable substance, and in certain cases could exert the pharmacological effects. The present study has demonstarted the effects of vitamin E supplementation on the oxidative stress in erythrocyte of the patients with NTDT. In this study, the amount of dietary intake prior to the study in the patients did not meet the Dietary Reference Intake for Thai (DRI). The results were similar to those found by Tanphaichitr *et al* (8) that children with thalassemia minor, intermedia and major had energy intake about 65% of the amount recommended for Thai children. Thalassemic patients are prone to malnutrition due to reduced nutrient absorption, increased nutrient losses, and increased nutrient metabolism (9). These conditions lead the patients to have increased requirements for certain nutrients. In the present study, although the caloric intake after 12-week vitamin E supplementation was not significantly different from baseline, average caloric intake of the patients in vitamin E group achieved the DRI for Thai. The increased total caloric intake may result from the increased proportion of carbohydrate intake in these children after the vitamin E supplementation.

This study showed that the levels of serum vitamin E at week 0 in the patients was lower than the normal range of healthy people (758–1952 μg/dL). This supported the results of the previous study by Kassab-Chekir *et al* (10) that vitamin E levels in serum of β-thalassemia patients decreased by 70%, compared to healthy children. Low level of vitamin E might be due to an excessive iron fraction that generated lipid peroxidation process resulted from oxidative stress (11), and thus the body required higher amount of antioxidant to scavenge these generated radicals. Vitamin E may play a role in neutralization of free radicals on red blood cell (RBC) membrane preventing erythrocyte membrane damage (12). Vitamin E deficiency manifests as a shortened half-life of erythrocytes, which can progress to increased hemolysis.

In the present study, serum vitamin E levels significantly increased after vitamin E supplementation for 12 weeks, whereas no change in serum vitamin E levels was observed in the control group. The serum vitamin E levels after supplementation of vitamin E were within the normal range of healthy people. The results were similar to those of previous studies. Suthutvoravut *et al* (13) found that plasma vitamin E levels in thalassemic children who had vitamin E deficiency increased to reach the normal range after supplementation with 200 mg of vitamin E for 4-8 weeks. Tesoriere *et al* (14) also found that oral treatment with 600 mg/d vitamin E for 3 months tended to normalize serum vitamin E levels in β-thalassemia intermedia patients. In addition, Dissayabutra *et al* (6) found that after supplementation of vitamin C (100 mg/d) and vitamin E (400-600 mg/d), plasma vitamin C and vitamin E levels significantly increased, even though they were still lower than the levels in normal population. Thus, vitamin E supplementation could increase serum

vitamin E levels and this may help reduce the progression and complications of disease.

Lipid peroxidation of erythrocytes is manifested by MDA production, which caused cross-linking of membrane components, leading to increased RBC membrane rigidity and decrease RBC deformability (15). Lipid peroxidation of cell membrane induced by cellular oxidative stress has been shown to be a key event in damage of RBC in patients with thalassemia (10). Several studies demonstrated that the MDA levels significantly increased in thalassemic patients (16-18). The present study found that supplementation of 10 IU/kg/d of vitamin E in thalassemic patients for 12 weeks produced significant decrease in MDA levels. The result was consistent with the previous study by Das *et al* (19) who found that treatment of the thalassemia major patients with vitamin E (10 IU/kg/d) for a period of 4 weeks remarkably reduced the level of lipid peroxidation in erythrocyte membranes. Significant decrease in MDA levels in vitamin E supplemented group was possibly due to improved status of vitamin E, which is a strong antioxidant nutrient. However, vitamin E supplementation may not always affect lipid peroxidation in thalassemic patients. George *et al* (20) evaluated the effect of vitamin E supplementation in hemoglobin H disease, and they found no change in MDA levels after supplementation with vitamin E at the dose of 400 IU/day for 2 months. The different results may be explained by the different duration, dose of vitamin E supplementation and severity of the diseases. The present study indicated that 12 weeks of vitamin E supplementation at the dose of 10 IU/kg/d was efficient in reducing MDA levels, and thereby protecting lipids from peroxidation by free radicals in NTDT patients.

Lipid peroxidation as well as the increase of cellular hydroxyl and related radicals has been long shown to induce hemolysis (21). This study revealed that in the control group the susceptibility of RBC to the H_2O_2-induced hemolysis increased over time, suggesting that vitamin E deficiency in the patients manifested a shortened half-life of erythrocytes and increased susceptibility to hemolysis. However, the percentage of hemolysis significantly decreased when RBC were treated with 50, 100 and 200 mM AAPH after vitamin E supplementation for 12 weeks. This result further supported the benefit of vitamin E supplementation that vitamin E not only prolonged RBC survival in the thalassemia major patients (42.8%) after administration of vitamin E (750 to 1000 IU/d) for 12 months (22) but also prevented hemolysis caused by oxidative stress induced by AAPH radical in NTDT patients. In addition, Suthutvoravut *et al* (13) found that patients with hemoglobin H disease had less degree of hemolysis after oral vitamin E supplementation at the daily dose of 6-14 mg/kg. In other hemolysis model using osmotic pressure, Palasuwan *et al* (23) evaluated the osmotic fragility of RBC in hemoglobin E carrier and found that the osmotic fragility of RBC significantly increased after vitamin E supplementation. The data revealed that the percentage of lysis of thalassemic red cells approached that of normal cells after 3-month administration of 200 IU of vitamin E. Vitamin E levels also showed positive correlation to red cell survival (20). These results indicated the protective effect of vitamin E against oxidative damage to the RBC membrane and the prolongation of RBC life span.

Conclusion

The present study provided information regarding the activity of vitamin E in attenuating oxidative stress in RBC of young patients with NTDT. Vitamin E supplementation for 12-week period was sufficient to reduce lipid peroxidation, and subsequently to suppress hemolysis. However, although the levels of vitamin E were improved and oxidative stress was reduced in the patients after vitamin E supplementation, no change in hematological parameters was observed. Therefore, further study is required. The duration of the experiments may be expanded to see more details in terms of mechanism of action as well as the defined clinical outcome.

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