RESEARCH NOTE

PRELIMINARY STUDY OF THE EFFECT OF VITAMIN E SUPPLEMENTATION ON THE ANTIOXIDANT STATUS OF HEMOGLOBIN-E CARRIERS

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Abstract. The antioxidant status of hemoglobin-E carriers was studied pre- and post-treatment with vitamin E for 3 months. Fourteen hemoglobin-E carriers (age = 21.36 ± 1.08 years, BMI = 18.32 ± 1.22 kg/m^2) were treated with vitamin E 200 I.U. daily for 3 months. Fasting blood samples were collected and analyzed for erythrocyte superoxide dismutase activity, total antioxidant activity, hemoglobin concentration, hematocrit, MCV, Heinz body formation and osmotic fragility test. The blood parameters before and after vitamin E treatment were compared. The results showed that superoxide dismutase activity in the erythrocytes was significantly decreased, while total antioxidant activity in plasma, and the osmotic fragility of the erythrocytes, was significantly increased after vitamin E supplementation. However, hematocrit, MCV, and Heinz body formation did not change significantly. This demonstrated that vitamin E 200 IU could be used as a lipophilic antioxidant in red blood cells and could help increase the level of antioxidant in hemoglobin-E carriers.

INTRODUCTION

Thalassemia syndrome is among the most common genetic diseases in Southeast Asian people. In Thailand, approximately 40% of the population are thalassemia carriers, which represents a public-health concern (Wasi, 1978). The syndromes are caused by an imbalance in the rate of synthesis of globin chains. The severity of globin chain impairment generates a diversity of thalassemic phenotypes. β-thalassemia arises as a consequence of a decrease or absent synthesis of the β-globin chain (Weatherall and Clegg, 1982; Kazazian and Boehm, 1988). As the result of altered β-globin chain biosynthesis, the concentration of $\alpha_2\beta_2$-hemoglobin tetramer (HbA) is substantially reduced, or absent. The excess pool of unpaired β-hemoglobin chains leads to oxidative erythrocyte damage, which might be further exaggerated by heme (Joshi *et al,* 1983).

Hemoglobin E trait is the most common form in northeastern Thailand (Wasi, 1978). Hemoglobin E results from the substitution of glutamic acid by lysine in the β-chain of hemoglobin and is mostly due to the interaction of β^o-thalassemia or β^+-thalassemia. β-thalassemia/Hb E shows a remarkable degree of variability in clinical expression, some of which are similar to homozygous β-thalassemia (Swarup et al, 1961; Frischer and Bowman, 1975). Iron overload is the consequence in the β-thalassemia/Hb E after several blood transfusions. Overloading of iron can generate a peroxidative status in β-thalassemia major and an increase in superoxide dismutase and erythrocyte glutathione peroxidase activity (Kassab-Chekir *et al*, 2003). Severe oxidative damage is observed in erythrocytes due to the presence of excess α-globin chains (Pearson *et al*, 1973). Thus, both the accumulation of excess α-chain and iron overload instigate increased red-blood-cell destruction, resulting in decreased antioxidants. In normal humans, free radicals are also produced by erythrocytes during the inflammatory response (Miyazaki *et al*, 2001). Whenever the antioxidant system is not adapted to excessive production of reactive radical oxygen species, oxidative stress is initiated. Although there is no clinical symptom in most hemoglobin-E carriers, the erythrocyte superoxide dismutase activity of β-thalassemia/Hb E carrier was found to be higher than healthy persons (Chakraborty and Bhattacharyya, 2001). In addition, our previous report showed that hemoglobin-E carriers had a low total antioxidant plasma level (Palasuwan *et al*, 2005). These show that the reactive radical oxygen species in hemoglobin-E carriers generate more oxidants in red blood cells and plasma than in healthy people, which implies that the imbalance between oxidants and antioxidants might affect the function of erythrocytes of hemoglobin-E carriers.

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Antioxidants are potentially protective agents that may help guard against oxidative hemolysis in thalassemia patients. Vitamin E is considered the most important lipid-soluble exogenous antioxidant in humans (Margaristis *et al,* 2003). Unchern and colleagues (2003) have reported that 3 months of daily vitamin E supplementation can significantly increase plasma α-tocopherol levels and reduce plasma oxidant levels in splenectomized β-thalassemia/Hb E patients. (Unchern *et al,* 2003). In this report, we suggest that vitamin E, a potent lipophilic antioxidant supplement, might help reduce oxidant levels in the blood of thalassemia carriers. We investigated hematological parameters: hematocrit, hemoglobin concentration, Heinz body formation, and osmotic fragility test, and total antioxidant status in plasma, and the enzymatic antioxidant defense system in the erythrocytes of hemoglobin-E carriers pre- and post-vitamin E 200 IU supplementation.

MATERIALS AND METHODS

Chemicals

Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) was purchased from Aldrich Chemical Co. 2,2´-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) diamonium salt (ABTS), potassium persulfate, xanthine, xanthine oxidase, nitroblue tetrasolium (NBT), sodium carbonate, superoxide dismutase from bovine, acetylphenylhydrazine, and ethylenediamine-tetraacetic acid (EDTA) were purchased from Sigma-Aldrich (St Louis, MO, USA), All other chemicals were of analytical grade and available locally.

Subjects and experimental protocol

Fourteen hemoglobin-E-carrier volunteers, aged 21.36 ± 1.08 years, were recruited from a campus population. Volunteers were of normal weight (body mass index, 18.81 ± 1.22 kg/m^2), and Hb E traits or carriers were diagnosed by hemoglobin electrophoresis analysis and also confirmed under the supervision of medical professionals in our laboratory.

All volunteers were non-smokers and none had renal or hepatic disease, diabetes, heart disease or hypertension, and had not taken antioxidant supplements for at least 6 months prior to the study. All volunteers ingested orally one tablet of vitamin E per day (Medicap product) during the 3-month experimental period. All volunteers were instructed before the beginning of the study carefully to comply with treatment and to take one tablet after breakfast. The protocol was approved by the Ethic Committee of Faculty of Pharmaceutical Sciences, Chulalongkorn University and all participants gave written by informed consent.

Blood sampling procedures

Venous blood samples were collected after overnight fast in the pre-treatment and post-treatment periods; the samples were collected in K_3EDTA tubes for complete blood count (CBC) and erythrocyte superoxide dismutase activity assay; as well as heparinized tubes (Li Heparin 500 U/10 ml) for Heinz body formation test, osmotic fragility test (OF), and total antioxidant capacity test. The hematocrit, hemoglobin concentration, mean corpuscular volume (MCV), Heinz body formation test, and osmotic fragility test were analyzed within 4 hours of venipuncture. Plasma samples were separated by centrifugation of the blood sample at 950*g* for 15 minutes within 30 minutes of venipuncture; then, they were kept at -40 °C until further analysis.

Biological analyses

Hematological parameters. Hematocrit, hemoglobin concentration, and MCV were determined by automated hematometer (Technicon H*3, Bayer).

Heinz body formation assay. Buffer solution, consisting of 1.3 parts of M/15 KH_2PO_4 (9.1 g of KH_2PO_4 dissolved in 1 liter deionized water) was mixed with 8.7 parts of M/15 Na_2HPO_4 (9.5 g of Na_2HPO_4 dissolved in 1 liter deionized water). Two hundred milligrams of glucose were added to 100 ml buffer solution.

Acetylphenylhydrazine solution was prepared by dissolving 100 mg of acetylphenylhydrazine in 100 ml buffer solution.

Crystal violet solution was prepared by dissolving 2 g of crystal violet powder in 100 ml of 0.73% NaCl at room temperature. The solution was shaken for 5 minutes, and then filtered through Whatman No.2 filter paper. The working solution was prepared by adding an equal volume of 0.73% NaCl solution to the filtered solution.

One hundred microliters of heparinized red cells were added to 2 ml of acetylphenylhydrazine. The mixture was incubated for 2 hours, and Heinz bodies counted. In the test system, each sample was tested in triplicate.

We performed the Heinz body counter stain by transferring solutions from each test and mixing with crystal violet solution at equal volumes. The mixture was left undisturbed at room temperature for 5 minutes. A thin smear was performed and Heinz bodies were observed in at least 1,000 red blood cells under a microscope (1,000 x) (Reinhart *et al*, 1986).

Osmotic fragility test. Fifty microliters of heparinized red blood cells were added to 5 ml of 0.36% sodium chloride solution. After 30 minutes of incubation, the supernatant was separated by centrifugation of the blood sample at 950*g* for 5 minutes. Then, the percentage hemolysis was detected by spectrophotometer (Spekol 1200, Analytik Jena AG) compared with blank (0.36% sodium chloride solution).

Total antioxidant activity assay. Total antioxidant activity was measured by radical cation decolorization assay (Miller *et al,* 1993; Re *et al,* 1999), which is based on absorbance inhibition by antioxidants of the free radical cation from ABTS (2,2´-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid diamonium salt). ABTS was incubated with potassium persulfate to produce the free radical cation ($ABTS^{o+}$). This had a relatively stable blue-green color, which was measured by spectrophotometer (Shidmadzu UV-1601) at 734 nm. Antioxidant compounds suppress the absorbance of $ABTS^{o+}$ to an extent on a time scale dependent on the antioxidant capacity in the plasma. This assay was calibrated using Trolox (a water-soluble vitamin-E analog) as standard.

In brief, ABTS was dissolved in deionized water to make a 7 mM concentration solution. $ABTS^{o+}$ was produced by mixing ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and the mixture was allowed to stand in the dark at room temperature for 12-16 hours before use. For our study, the $ABTS^{o+}$ solution was diluted with PBS, pH 7.4, to an absorbance of 0.70 (±0.02) at 734 nm. After addition of 1.0 ml of diluted $ABTS^{o+}$ ($A_{734nm} = 0.700 \pm 0.200$) to 10 µl of each heparinized plasma or Trolox standard (final concentration 0-15 µM) in PBS, the absorbance reading was taken at 30 °C exactly 6 minutes after initial mixing. A PBS blank was run in each assay. All determinations were carried out at least in triplicate. The percentage absorbance inhibition at 734 nm was calculated and plotted as a function of antioxidants of Trolox for the standard reference data.

Erythrocyte superoxide dismutase activity. Plasma was removed by centrifugation of the EDTA blood sample at 3,000*g* at 4 °C for 10 minutes. After separation, the packed red blood cells (RBCs) were collected and then washed 3 times with 2 volumes of isotonic saline solution at pH 7.0. The washed RBCs were then hemolysed by suspension in double-distilled water, and centrifuged at 15,000 *g* for 40 minutes. The supernatant representing the hemolysate was kept at 70 °C until analysis.

Erythrocyte superoxide dismutase was detected by enzyme kinetic assay (Suttle and McMurray, 1983; Arthur and Boyne, 1985). In brief, 5 µl of hemolysate were added to the mixed substrate (xanthine 0.05 mM, NBT 0.025 mM, EDTA 0.94 mM, and sodium carbonate buffer pH 10.2). Then, 20 µl of xanthine oxidase were added to the reacting well. Erythrocyte superoxide dismutase was detected by spectrofluorometer (Perkin Elmer 1420 Multilabel Counter) at 560 nm.

Statistical analysis

All parameters were expressed as mean ± SD, and statistically significant differences between values were obtained using paired-sample *t*-test. The criterion for significance was $p<0.05$.

RESULTS

The hematological parameters, hemoglobin concentration, hematocrit, MCV, Heinz body formation, osmotic fragility, and total antioxidant status in plasma, and erythrocyte superoxide dismutase in hemoglobin-E carriers pre- and post-vitamin-E 200 IU supplementation are presented in Table 1. The pre- or post-supplementation values for hemoglobin, hematocrit, MCV, and Heinz body formation did not change. However, the osmotic fragility of red blood cells and total antioxidant capacity in plasma increased significantly, while Heinz body formation and erythrocyte superoxide dismutase decreased significantly.

DISCUSSION

We investigated the antioxidant status of thalassemic erythrocytes by direct measurement of cytoprotective enzyme. During normal cellular metabolism, cellular defenses consist of cytoplasmic enzymes. A response to oxidative stress perturbs the normal cellular metabolism, including an increase in intracellular free calcium, concentration damage to membrane ion transporter proteins, and peroxidation of lipids. Superoxide dismutase is an important antioxidant enzyme, functioning in the elimination of superoxide radicals (O^-_2). Superoxide dismutase changes the free radical to H_2O_2, which will be transformed to water by catalase enzyme (Chakraborty and Bhattacharyya, 2001). The decreased catalytic activity of superoxide dismutase might be involved in reduced scavenging of the superoxide radical (O^-_2), thereby producing less hydrogen peroxide in the erythrocytes.

Many studies have shown that hemoglobin-E carriers have more superoxide dismutase activity. This implies that hemoglobin-E carriers must have

Table 1
Hematological parameters and antioxidant activity (mean ± SD) in hemoglobin-E carriers before and after vitamin E supplementation.

Test	Before vitamin E supplementation	After vitamin E supplementation
Hemoglobin concentration (mg %)	12.29 ± 1.73	12.41 ± 1.57
Hematocrit (%)	38.94 ± 3.46	38.90 ± 4.92
Mean Corpuscular Volume (femtoliters)	76.89 ± 9.28	75.88 ± 8.69
Osmotic fragility (%)	64.23 ± 8.08	71.40 ± 8.51[a]
Heinz body formation (%)	98.57 ± 1.74	97.85 ± 1.75
Total antioxidant capacity (mM Trolox equivalent)	2.13 ± 0.94	2.25 ± 0.05 [a]
Superoxide dismutase (unit / g Hb)	14,043.86 ± 3,875.00	8,188.55 ± 3,473.32 [b]

[a] significantly increased (p = 0.05), [b] significantly decreased, (p = 0.05)

more free radicals within their red cells than healthy persons. However, catalase activity does not increase with increasing levels of free radicals (Chakraborty and Bhattacharyya, 2001). It is possible that H_2O_2 might be inside the hemoglobin-E red blood cells and might damage red cell walls by lipid peroxidation. Since hemoglobin-E carriers have more free radicals inside their red cells, the possibility of globin precipitation for these patients would be higher (Frischer *et al*, 1975). Moreover, total antioxidant levels in the plasma of hemoglobin-E carriers are low, probably because of the high usage rate (Palasuwan *et al*, 2005). Although hemoglobin-E carriers do not appear pale, they are prone to the risk of red-cell and tissue damage by oxidants. It is generally known that consumption of vitamin E, an antioxidant found inside cells, can help reduce the rate of cell-wall-lipid peroxidation (Emma *et al*, 2001; Della Rovere *et al*, 2004). In addition, high-dose vitamin E, 400-1200 IU is used in the treatment of many diseases, such as thalassemia, cancer, heart disease, diabetes, and renal disease (Morris and Carson, 2003; Das *et al*, 2004; Forman and Altman, 2004; Ong-awyooth *et al*, 2004; Panagiotis *et al*, 2005) without any side effects (Morinobu *et al*, 2002).

In this study, we investigated the effect of vitamin E 200 IU on hemoglobin-E carriers; this is a preventive dose that could be taken daily to help boost immune response. We found that after daily consumption of vitamin E 200 IU for 3 months, superoxide dismutase activity decreased significantly. Total antioxidant capacity in our hemoglobin-E carriers also showed a significant increase, which indicated an elevation in the oxidative defense mechanism to neutralize the damaging effects of oxidative species (Cighetti *et al*, 2002; Kassab-Chekir *et al*, 2003). We concluded that vitamin E 200 IU could help reduce the oxidant species inside red cells and increase the plasma antioxidant level in Hb-E carriers. However, there were no changes in hemoglobin concentration, hematocrit, or MCV. Heinz body formation by phenylhydrazine challenging method was used to test red cell endurance to oxidative stress (Johnston and Cox, 2001). Our study found that vitamin E 200 IU could not help reduce Heinz-body formation in thalassemia carriers, possibly because the dose was not high enough to protect red cells from severe oxidative stress. However, the previous result supported the suggestion that high-dose vitamin E administration in splenectomized β-thalassaemia/Hb E patients could reduce oxidative stress (Unchern *et al*, 2003). Moreover, Johnston and Cox (2001) reported the antioxidant effect of another antioxidant supplement, vitamin C, and found that the dosage that helps reduce Heinz-body formation must be as high as 575-1,075 mg /day.

The one tube osmotic fragility test (0.36% NaCl) was used to examine red cell ability to endure hypotonic solution. In healthy people, red cells would lyse ≥ 85% at 0.36% NaCl solution, while the percentage red-blood-cell lysis would be less in thalassemia carriers (Pootrakul *et al*, 1978), due to the higher surface-to-volume ratio of thalassemic red cells (Schrier *et al*, 1989). It was also reported that the red cell membranes of thalassemia patients change due to cross-linking of malondialdehyde, the product of lipid peroxidase reaction (Kahane *et al*, 1978). It was very interesting that the percentage of lysis of thalassemic red cells was approaching that of normal red cells after 3-months' administration of 200 IU vitamin E. Further investigation is needed to clarify whether this was the effect of vitamin E on red cells or the lower amount of malondialdehyde produced in red cell membranes.

Finally, we can conclude that vitamin E 200 IU could be used as a lipophilic antioxidant in red blood cells and could help increase the level of antioxidants in hemoglobin-E carriers.

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