

ANTIOXIDANT PROTECTION IN HEMOGLOBIN E TRAIT SUBJECTS AFTER VITAMIN E SUPPLEMENTATION

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Abstract. Hemoglobin E trait is the most common form of thalassemia in Thailand. Thalassemia carriers have impaired biosynthesis of globin leading to an imbalance of globin chains, resulting in damaged red blood cells, decreased red cell life span, and the generation of oxygen-free radicals. Vitamin E, a potent antioxidant, may help with these abnormalities because of its antioxidant status. Two hundred international units of vitamin E was given daily for three months to 30 hemoglobin E trait subjects and 28 matched healthy subjects. Erythrocyte superoxide dismutase, erythrocyte glutathione peroxidase, total plasma antioxidant activity, lipid peroxidation, hemoglobin, hematocrit, mean corpuscular volume and total plasma vitamin E were determined for each subject. Superoxide dismutase and glutathione peroxidase in erythrocytes, as well as the total antioxidant activity and the level of vitamin E in the plasma of hemoglobin E trait subjects and healthy subjects increased significantly ($p < 0.01$). Lipid peroxidation (MDA) decreased significantly ($p < 0.01$), and hemoglobin, hematocrit and MCV did not change significantly ($p > 0.05$).

INTRODUCTION

Thalassemia is a public health concern in Southeast Asia and India. Approximately 40% of the Thai population are thalassemia carriers (Wasi, 1978). It is caused by an imbalance in globin chain synthesis. The severity of impairment in globin chain synthesis results in the diversity of thalassemic phenotypes (Weatherall and Clegg, 1982; Kazazian and Boehm, 1988). Hemoglobin E is the most common form of thalassemia in Thailand and is caused by the substitution of glutamic acid by lysine at codon 26 of the β -globin chain. This mutation also activates a cryptic mRNA splice site, which results in reduced synthesis of the β -E chain and leads to a thalassemia phenotype (Swarup *et al*, 1961; Frischer and Bowman, 1975; Vichinsky, 2007).

Under normal conditions, cells have antioxidant protection mechanisms, including a number of antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT), as well as nonenzymatic antioxidants, such as glutathione (GSH), protein -SH, ascorbic acid, and uric acid, to protect themselves from the toxicity of reactive oxygen species (Halliwell and Gutteridge, 1986; Ha, 2004; Halliwell, 2006; Rizvi and Maurya, 2007). Whenever the antioxidant system is unable to adapt to excess production of reactive radical oxygen species, the oxidative stress is initiated. The presence of excess α -globin chains leads to erythrocyte damage by reactive oxygen species (Joshi *et al*, 1983). In either β -thalassemia major or β -thalassemia Hb E, iron overload is a common consequence of blood transfusions. Iron overload can generate peroxidation, and increase superoxide dismutase and erythrocyte glutathione peroxidase activity (Kassab-Chekir *et al*, 2003). Thus, accumulation of excess α -chains and iron overloaded may contribute to red blood cell destruction and resulting in decreased antioxidants protection. Hb E has a weakened α / β interface, leading to instability

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during conditions of increased oxidative stress (Vichinsky, 2007). Although Hb E trait has no clinical significance, patients may have mild microcytosis. The erythrocyte superoxide dismutase activity of β -thalassemia Hb E carriers is significantly different from healthy persons (Chakraborty and Bhattacharyya, 2001). Our previous study showed that hemoglobin E carriers have lower total antioxidant capacity in plasma (Palasuwan *et al*, 2005). This means reactive radical oxygen species in hemoglobin E carriers generate more oxidants in red blood cells and plasma than in healthy people. It implies that an imbalance in pro-oxidants and antioxidants may affect the function of erythrocytes in hemoglobin E carriers.

Vitamin E, an important lipid-soluble exogenous antioxidant in humans, has been used as a potential agent to help protect against oxidative stress in thalassemia patients (Das *et al*, 2004; Della Rovere *et al*, 2004). Unchern and colleagues (2003) reported that three months of daily vitamin E supplementation can significantly increase plasma α -tocopherol levels and reduce plasma oxidant levels in splenectomized β -thalassemia/HbE patients. In our previous preliminary study, we demonstrated the administration of vitamin E for 3 months was able to increase total antioxidant capacity in plasma and reduce erythrocyte rigidity in thalassemia carriers (Palasuwan *et al*, 2006). Thus, we investigated

other parameters, including glutathione peroxidase and superoxide dismutase, in erythrocytes, as well as α -tocopherol, total antioxidant activity and a lipid peroxidation index (malondialdehyde) in the plasma of hemoglobin E trait subjects before and after vitamin E 200 IU supplementation.

MATERIALS AND METHODS

Subjects

Two hundred volunteers were enrolled from a university campus. All were non-smokers, and none had renal disease, hepatic disease, diabetes, asthma, cancer, heart disease, or hypertension. None had taken dietary or antioxidant supplements for at least one year prior to this study. All were diagnosed as being thalassemia Hb E carriers by hemoglobin typing, DCIP analysis and confirmed by medical professionals in our laboratory. The study was approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University. All subjects were informed of nature of the experiment before giving informed consent. Thirty-two hemoglobin E carriers (10 males, 22 females) were selected and compared with 30 matched healthy volunteers (8 males, 22 females). The selected subjects were instructed before beginning the study to carefully comply with the treatment and to take an oral tablet of

Table 1
Selected characteristic of all subjects who completed the study protocol.

	Hemoglobin E trait subjects (<i>n</i> = 30)	Healthy control subjects (<i>n</i> =28)	All subjects (<i>n</i> =58)
Age (yrs)	22 \pm 4	25 \pm 10	23 \pm 7
BMI (kg/m ²)	20 \pm 3	20 \pm 3	20 \pm 3
Hemoglobin (g/dl)	12.2 \pm 1.7	12.3 \pm 1.9	12.2 \pm 1.8
Hematocrit (g%)	39 \pm 5	41 \pm 6	40 \pm 6
Mean corpuscular volume (fl)	77 \pm 9 ^a	91 \pm 8	84 \pm 11

^aSignificant difference (*p*<0.05), present as mean \pm SD

vitamin E 200 IU (Medicraft product, Bangkok, Thailand) daily after breakfast for the 3-month experimental period. Four subjects (2 male subjects) with Hb E trait and 2 healthy female subjects) left the study because they could not complete the protocol. The characteristics of the subjects are presented in Table 1.

Blood sampling

Venous blood samples were collected pre- (one day before supplementation), mid- (Day 45) and post- (Day 90) treatment. Blood samples were drawn from a forearm vein and collected in a K3EDTA tube (6 ml) (Vacuette™, Greiner Bio-One, Germany) after an overnight fast. A hematocrit, hemoglobin concentration, and MCV were analyzed within 2 hours of venepuncture. Plasma was removed by centrifugation at 3,000g at 4 °C for 15 minutes within one hour of collection. After separation, the packed red blood cells (RBCs) were collected and washed three times with isotonic saline solution at pH 7.2. The washed RBCs were then hemolyzed by suspending in double distilled water and centrifuged at 3,000g at 4 °C for 15 minutes. In each assay, using hemolysate or plasma, was performed in triplicate the same day to eliminate variation in assay conditions. Blood samples were stored in eppendorf™ tubes at - 80 °C and thawed only once before analysis to eliminate freeze-thaw effect.

Hematological parameters

Hematocrit, hemoglobin concentration, and MCV were determined by an automated hematology analyzer (Technicon H*3, Bayer, USA).

Plasma α -tocopherols

The concentration of α -tocopherol in the plasma was assayed as described by Ueda and Igarashi (1987) using high-performance liquid chromatography (Shimadzu LC-10AS Class VP, Japan). The detection wavelengths used were excitation at 297 nm and emission at 327 nm. The analytical column Develosil (normal phase) was used (250 mm x 4.5 mm i.d.).

Total antioxidant status assay (TAS)

The total plasma antioxidant status was measured using a radical cation decolorization assay (Miller *et al*, 1993; Re *et al*, 1999). This assay was based on the inhibition by antioxidants of the free radical cation from ABTS (2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid diammonium salt) (Sigma-Aldrich, USA). ABTS was incubated with potassium persulfate (Sigma-Aldrich, USA) in order to produce a free radical cation (ABTS^{•+}). This had a relatively stable blue-green color, which was measured by spectrophotometer (Shimadzu UV-1601, Japan) at 734 nm. This assay was calibrated using Trolox, a water-soluble vitamin E analog (Aldrich Chemical, USA) as standard.

Index of lipid peroxidation

Lipid peroxidation was assayed by measuring formation of malondialdehyde (MDA) (Satoh, 1978), using thiobarbituric acid (TBA) (Sigma-Aldrich, USA) and trichloroacetic acid (TCA) (Fluka and Riedel, Germany). MDA was extracted using n-butyl alcohol and revealed at 530 nm by spectrophotometer (Shimadzu UV-1601, Japan) and quantified by reference to a calibration curve for tetraethoxypropane (Sigma-Aldrich, USA).

Erythrocyte superoxide dismutase (SOD) and erythrocyte glutathione peroxidase (GPx)

Erythrocyte superoxide dismutase was detected from hemolysate by an enzyme kinetic-colorimetric assay (Arthur and Boyne, 1985; Suttle, 1986) using a kit from Randox Laboratories, UK. Glutathione peroxidase was assayed using the method of Paglia and Valentine (1967) from hemolysate with an enzyme kinetic-colorimetric kit from Randox Laboratories, UK.

Statistical analysis

Statistical analysis was performed using the program SPSS, version 15.0. All data are presented as mean \pm standard deviation.

An independent t-test was used to compare the values of Hb E trait subjects with the healthy subject group at baseline (1 day before treatment). Comparisons were made using the one-way ANOVA test (supplementation) with repeated measures (pre-, mid-, and post-treatment) to determine (1) the differences between the supplemented and control groups over time (vitamin E intake x genetic inheritance x duration), the differences between the supplemented and control groups (vitamin E intake x genetic inheritance), the effect of supplementation over time (vitamin E intake x duration). The Mauchly's test was used to test

for sphericity, while the Greenhouse-Geisser, Huynh-Feldt, and Lower-bound were used for those variables that did not follow sphericity. When significant changes were observed in ANOVA tests, a post hoc test-LSD was applied to locate the source of significant differences.

RESULTS

The hematological values (hemoglobin concentration, hematocrit and MCV) are presented in Table 2. Plasma α -tocopherol, total antioxidant status (TAS), lipid peroxidation index (MDA), erythrocyte superoxide

Table 2
Hematological parameters in each group.

	Hb E trait subjects (n=30)		Healthy subjects (n=28)	
	Pre-supplementation	Post-supplementation	Pre-supplementation	Post-supplementation
Hemoglobin (g/dl)	12.2 ± 1.7	12.4 ± 1.5	12.3 ± 1.9	12.7 ± 1.5
Hematocrit (g%)	38.7 ± 5.2	38.4 ± 4.5	41.1 ± 5.7	39.9 ± 5.1
Mean corpuscular volume (fl)	77.2 ± 9.0	75.8 ± 9.5	91.4 ± 7.8	88.0 ± 8.5

Present as mean ± SD

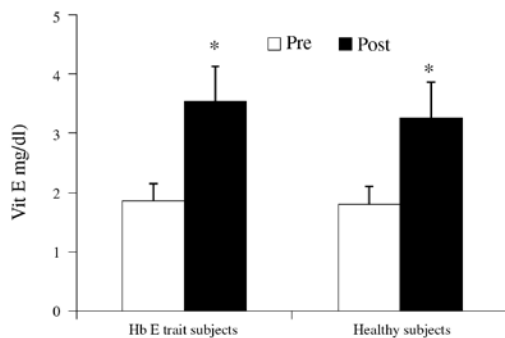


Fig 1- Plasma α -tocopherol (Vit E; mg/dl) in Hb E trait subjects (n=30) and healthy control subjects (n=28). At baseline, mean values of vitamin E levels in Hb E trait subjects and healthy control subjects were not different (p>0.05). Mean values (within-group) post-treatment were significantly different from pre-treatment in both study groups (*p<0.001). The rates of elevation of α -tocopherol in the Hb E trait subjects and the healthy control subjects were not different (p>0.05).

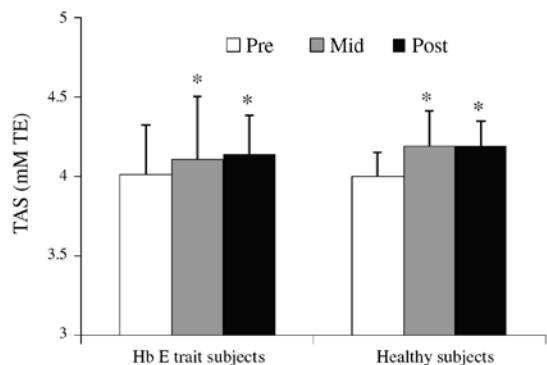


Fig 2- Total antioxidant status (TAS; mM trolox equivalence) in Hb E trait subjects (n=30) and in healthy control subjects (n=28). At baseline, the mean values for TAS in the Hb E trait subjects and the healthy control subjects were not different (p>0.05). The mean values (within-group) for mid- and post-treatment were significantly different from pre-treatment in both study groups (*p<0.001). The rate of elevation of TAS in the Hb E trait subjects and healthy subjects were not different (p>0.05).

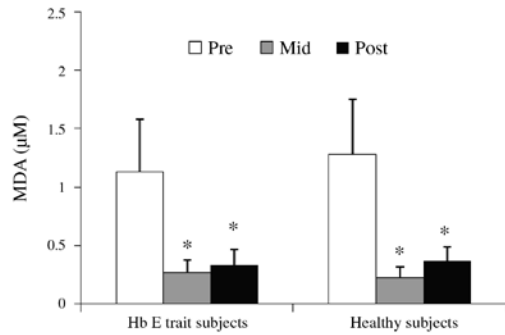


Fig 3- Lipid peroxidation index (MDA; μM) in Hb E trait subjects ($n=30$) and in healthy control subjects group ($n=28$). At baseline, mean values for MDA in Hb E trait subjects and healthy control subjects were not different ($p>0.05$). Mean values (within-group) for mid- and post-treatment were significantly different from pre-treatment in both subject groups ($*p<0.001$). The rate of reduction of MDA in Hb E trait subjects and healthy subjects were not different ($p>0.05$).

dismutase (SOD), and erythrocytes glutathione peroxidase (GPx) are presented in Figs 1, 2, 3, 4, and 5, respectively. The hematological values did not change significantly between before and after supplementation. The level of vitamin E, total antioxidant status, superoxide dismutase and glutathione peroxidase in the hemoglobin E trait subjects and healthy controls increased significantly ($p<0.001$), and the lipid peroxidation (MDA) decreased significantly ($p<0.001$).

DISCUSSION

Under normal cellular metabolism, endogenous antioxidant protection consists of cytoprotective enzymes, such as superoxide dismutase, catalase and glutathione peroxidase. In this study, we investigated the antioxidant status of hemoglobin E carriers and healthy controls by measurement of exogenous vitamin E in plasma (α -tocopherol), endogenous enzymes in erythrocytes (SOD and GPx), lipid peroxidation product (MDA) and the total antioxidant status (TAS) in the plasma. Superoxide dismutase catalyzes dismutation of superoxide radicals to hydrogen peroxide

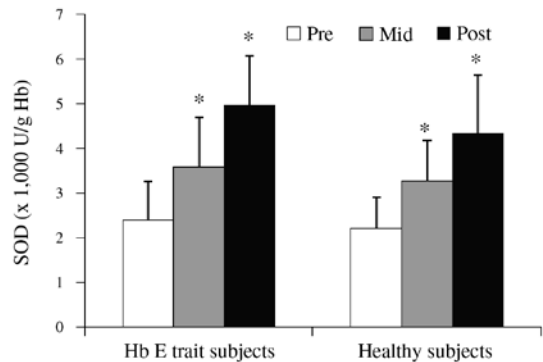


Fig 4- Erythrocyte superoxide dismutase (SOD; U/g Hb) in Hb E trait subjects ($n=30$) and in healthy control subjects ($n=28$). At baseline, mean values in Hb E trait subjects and in healthy control subjects were not different ($p>0.05$). Mean values (within-group) for mid- and post-treatment were significantly different from pre-treatment in both studied groups ($*p<0.001$). The rate of elevation of SOD in Hb E trait subjects and healthy control subjects were not different ($p>0.05$).

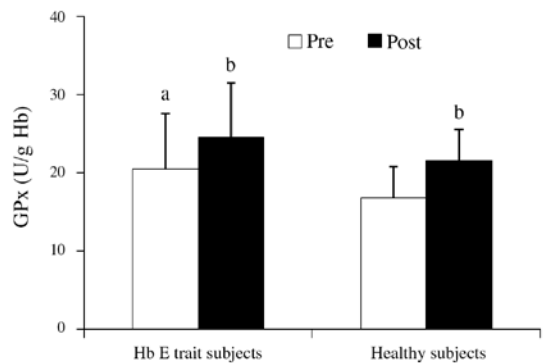


Fig 5- Erythrocyte glutathione peroxidase (GPx; U/g Hb) in Hb E trait subjects ($n=30$) and in healthy control subjects ($n=28$). At baseline, the mean values for GPx in Hb E trait subjects and healthy control subjects were different ($*p=0.027$). Mean values (within-group) for post-treatment were significantly different from pre-treatment in both studied groups ($*p<0.001$). The rate of elevation of GPx in Hb E trait subjects and in healthy control subjects were not different ($p>0.05$).

which is transformed into water by catalase (Chakraborty and Bhattacharyya, 2001). Glutathione peroxidase detoxifies hydrogen peroxide and converts lipid hydroperoxides into nontoxic alcohols (Czernichow and Hercberg, 2001). Lipid peroxidation is believed to be an

important cause of damage to and destruction of cell membranes and has been suggested to be a contributing factor to the development of oxygen radical-mediated tissue damage (Kaya *et al.*, 1999). In our study, glutathione peroxidase activity was significantly higher in Hb E trait subjects compared to healthy control subjects at baseline before supplementation ($p=0.028$), suggesting erythrocytes may have a protective mechanism to reduce the pro-oxidant condition, which may cause increased hemolysis and microcytosis observed as the lower MCV in Hb E trait patients ($p<0.001$).

Vitamin E, an essential fat-soluble vitamin, plays a crucial role in the endogenous defense against peroxidation of the membrane lipid at an early stage through free radical-quenching activity (Kokcam and Naziroglu, 2002). Vitamin E at 200 IU taken daily may help boost the immune response. We found that after consumption of vitamin E 200 IU daily for three months, the level of α -tocopherol and the activity of glutathione peroxidase and superoxide dismutase were increased in both Hb E carriers and in control subjects ($p<0.001$). It may be inferred that α -tocopherol status in the blood was contributing greater protection after treatment, as observed in the significantly higher vitamin E level ($p<0.001$). This then led to an increase in glutathione peroxidase and superoxide dismutase, which changed the hydroperoxide group to the much less toxic hydroxyl free radical. The rate of lipid peroxidation released from the lipid membrane of erythrocytes into the plasma may be lower since we see of the lower MDA index ($p<0.001$) and improvement in total antioxidant capacity of the plasma ($p<0.001$). Compared to control groups, all changing rates of TAS, SOD, GPx, MDA and TAS in Hb E trait subjects were not significantly different from healthy subjects ($p>0.001$). This may imply that after vitamin E supplementation the antioxidant protection mechanism in hemoglobin E trait subjects was not different from that of healthy persons.

In conclusion, daily supplementation with 200 IU of vitamin E may help increase antioxidant protection and reduction of lipid peroxidation in healthy subjects and in those with hemoglobin E trait.

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