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Polish Academy of Sciences

Polish Journal of Pharmacology
Pol. J. Pharmacol., 2004, 56, 85–96
ISSN 1230-6002

ATTENUATION OF OXIDATIVE STRESS-INDUCED CHANGES IN THALASSEMIC ERYTHROCYTES BY VITAMIN E

Nandita Das[1], *Tapasi Das Chowdhury*[1], *Aindrila Chattopadhyay*[2], *Asoke G. Datta*[1#]

[1]Department of Life Science and Biotechnology, Jadavpur University, Kolkata - 700 032 India;
[2]Present address: Department of Physiology, Vidyasagar College 39 Sankar Ghosh Lane, Kolkata -700 006, India

Attenuation of oxidative stress-induced changes in thalassemic erythrocytes by vitamin E. N. DAS, T. DAS CHOWDHURY, A. CHATTOPADHYAY, A.G. DATTA Pol. J. Pharmacol. 2004, 56, 85–96.

The oxidative stress status of the transfusion-dependant Eβ- and β-thalassemia patients were studied before and after treatment with vitamin E for a period of four weeks. The level of cellular vitamin antioxidants *viz*. ascorbic acid and vitamin E in the thalassemia patients were found to be considerably lower compared to normal subjects. The activities of enzymatic antioxidants *viz*. catalase, glutathione peroxidase and glutathione reductase were found to be drastically reduced in untreated Eβ- and β-thalassemic patients when compared to normal subjects. However, the activity of superoxide dismutase was found to be increased in both types of untreated thalassemic patients when compared to normal individuals. An increase in superoxide dismutase and a decrease in catalase activity reflects the presence of a severe oxidative stress situation in the erythrocytes of the untreated transfusion dependant Eβ- and β-thalassemia patients. Changes in erythrocyte membrane protein pattern in untreated Eβ- and β-thalassemia patients when compared to normal erythrocyte further confirm the presence of continued oxidative stress in the ailing thalassemic erythrocytes. All these changes in the antioxidant status as well as the changes in the erythrocyte membrane proteins are ameliorated to considerable extent when the transfusion-dependent Eβ- and β-thalassemia patients were treated with vitamin E at a dose of 10 mg/kg /day for a period of four weeks. The patients during the treatment period did not exhibit any side effects and gained in body weight indicating a healthy status. The present study reveals that the lipophilic antioxidant vitamin E could be useful in the management of transfusion-dependant Eβ- and β-thalassemia patients.

Key words: *thalassemia, erythrocyte, oxidative stress, antioxidant defense, antioxidant, vitamin E*

correspondence; e-mail: dbandyo_5@yahoo.com; asoke_datta@yahoo.com

Abbreviations: DTNB – *5,5'-dithio-bis-nitrobenzoic acid,* EDTA – *ethylenediaminetetraacetic acid, tetrasodium salt,* GR – *glutathione reductase,* GSH-Px – *glutathione peroxidase,* H_2O_2 – *hydrogen peroxide,* MDA – *malondialdehyde,* NADP – *nicotinamide adenine dinucleotide phosphate***,** NADPH – *nicotinamide adenine dinucleotide phosphate reduced form***,** O_2^- – *superoxide anion,* $OH^\bullet$ – *hydroxyl radical,* PHX – *phenylhydrazine hydrochloride***,** TBARS – *thiobarbituric acid reactive substances***,** TEMED – *N,N,N',N'- tetramethylethylenediamine*

INTRODUCTION

Thalassemia is a severe, usually fatal form of anemia. About 190 millions of people throughout the world carry a hemoglobinopathy gene and more than 90 millions of people carry defective genes leading to thalassemia. Beginning approximately two to four months after birth, the disease is associated with profound anemia, jaundice, splenomegaly, expanded marrow space, siderosis and cardiomegaly. Impaired erythropoiesis, erythrocyte hemolysis in the peripheral circulation and deposits of excess iron in the tissues are a few causes of the abnormalities seen in thalassemia [1].

Generally, there is a genetically determined reduction in the rate of synthesis of one or more types of normal hemoglobin (Hb) polypeptide chains. When the defect is in the production of β-chain, the disease is β-thalassemia and similarly, a defect in the α-chain synthesis is called α-thalassemia. Therefore, the abnormalities of erythrocytes of thalassemiacs result from the accumulation of the unmatched normal globin chains that are present in excess [1].

A third type of thalassemia known as Hb Eβ-thalassemia is found exclusively in South-East Asia. It is a heterozygote state in which the gene for abnormal Hb E (which is a β-chain variant) is inherited from one parent and β-thalassemia from the other parent. The interaction of Hb E with β-thalassemia gene precipitates Eβ-thalassemia syndrome. Like normal Hb A, Hb E has two α- and two β-chains. The two α-chains have no abnormality. The abnormality resides in the β-chain and is demonstrable by tryptic digestion of globin. It was found that in Hb E, the normal glutamyl residue in the 26th position of the β-chain is substituted by a lysyl residue [11].

It is generally assumed that the excess of unpaired globin chains coalesce to form Heinz bodies which in turn induce extensive membrane damage. The Heinz bodies tend to precipitate in the cell and associate with various components of erythrocyte membrane. Eventually, they disintegrate to heme and globin moieties, loading the erythrocyte membrane with denatured globin chains, heme and iron [41]. Earlier studies have shown that thalassemic erythrocytes are exposed to higher oxidative stress [23], and a possible consequential accelerated apoptosis [27] contributing to shortened life span, as a result of excess production of reactive oxygen intermediates such as superoxide anion (O_2^-), hydroxyl radical ($OH^\bullet$), singlet oxygen and hydrogen peroxide (H_2O_2) within the erythrocytes. That these reactive intermediates then eventually oxidize various erythrocyte components including membrane proteins and lipids is indicated by 50% lowering of titrable-SH groups [23], while lipid oxidation was suggested by the lower ratio of unsaturated to saturated fatty acids of membrane lipids [38]. In addition, malondialdehyde (MDA), a product of lipid peroxidation, is generated in excess amounts in thalassemia, supporting the fact that large amounts of membrane bound iron are present in thalassemic erythrocytes. MDA is a bifunctional reagent and has been reported to crosslink several cell constituents including membrane components [24]. A crosslinked erythrocyte membrane is expected to be rigid and this could probably explain the rigidity of thalassemic erythrocytes when compared to normal ones. Further, erythrocyte deformability is a major determinant of anemia in thalassemia [17].

The present study systematically provide evidence, perhaps for the first time, that erythrocytes of the patients suffering from transfusion-dependent Eβ- and β-thalassemia continue to remain under severe oxidative stress as is reflected by increased lipid peroxidation of the erythrocyte membrane, increased protein oxidation as measured by tyrosine release, disturbed antioxidant defense system as well as altered protein pattern as reflected by SDS-PAGE analysis of the membrane proteins. All these changes, as the present investigation demonstrates, are attenuated to some extent on treatment of the thalassemic patients with a lipophilic antioxidant, *viz.* vitamin E, thereby strongly indicating that this vitamin could be potentially important in the management of transfusion-dependent Eβ- and β-thalassemic patients.

MATERIALS and METHODS

Materials

Thiobarbituric acid (TBA), glycine, tyrosine, ferric chloride and phenylmethylsulfonyl fluoride (PMSF) were purchased from E. Merck Lmt., Mumbai, India. Adenosine triphosphate (ATP), nicotinamide adenine dinucleotide phosphate reduced form (NADPH), nicotinamide adenine dinucleotide phosphate (NADP), bovine serum albumin (BSA), catalase, glutathione, glutathione reductase (GR), phenylhydrazine hydrochloride (PHX), Tris, ethylenediaminetetraacetic acid, tetrasodium salt (EDTA), manganese hydrochloride, mercaptoethanol, 2,6-dichlorophenolin-dophenol (DCIP) bromophenol blue and Coomassie blue G-250 and R-250 were procured from Sigma Chemical Company, St. Louis, MO, USA. 5-5'-Dithio-bis-nitrobenzoic acid (DTNB), ethylene dichloride, acrylamide, N-N'-bis acrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium persulfate (APS) were procured from Sisco Research Laboratory Pvt. Ltd., Mumbai, India. Other reagents used were of analytical grade.

Methods

Vitamin E therapy to thalassemic patients undergoing transfusion: 18 patients (9 Eβ-thalassemia and 9 β-thalassemia patients) from Indian Association of Blood Cancer and Allied Diseases (IABCD), Kolkata, India, were selected irrespective of sex. Thalassemic patients whose parents gave written consent to the present study were considered. These patients were undergoing transfusion and treatment at the IABCD, Kolkata, India. The Director of the organization also consented to the present study. Finally, this study was approved by the Ethics Committee of Jadavpur University. Patients age of the patients ranged from 4 years to 16 years and they received transfusion at every three to four weeks interval. Blood was drawn from the patients each time just before they received transfusion when hemoglobin values were at their lowest. In this way we could assess the actual condition of the patients before they received exogenous blood. Eight normal individuals were selected from our laboratory irrespective of sex.

The vitamin E therapy in thalassemic patients started immediately after the first transfusion (after selection of the patients). The patients were given vitamin E (Evion, E. Merck Ltd., Mumbai, India) at a dose of 10 mg/kg per day for 4-weeks and blood was taken on 29th day of the experiment and isolation of erythrocyte as well as erythrocyte lysate and the membrane preparation was carried out as described below. With these preparations lipid peroxidation, proteolytic and antioxidant enzyme activities were determined. SDS-PAGE analysis of erythrocyte cytoskeletal proteins were carried out before and after vitamin E therapy. Vitamin C and E content of plasma and vitamin E content of erythrocytes were also analyzed as described below.

Isolation of erythrocytes: normal human blood in citrate-phosphate-dextrose buffer was collected from Hemophillia Society, Kolkata and IABCD, Kolkata, India. These institutions are registered blood banks and transfusion centers. The packed red cells were obtained by centrifugation at 1000 × g for 10 min at 4°C. The plasma and the buffy coat were removed by aspiration and the red cells thus obtained were washed three times in 0.9% saline.

Treatment of erythrocytes with the oxidant

Fifty ml of a 20% suspension of washed erythrocytes were treated with a solution of 2 mM PHX (final concentration) and the mixture was kept at 37°C for 1 h. The erythrocytes were then washed thrice with saline prior to lysis and preparation of membrane therefrom.

Preparation of hemolysate from thalassemic erythrocytes for the measurement of proteolytic activity and assay of antioxidant enzymes

Four and a half ml of blood was collected from the thalassemia patient in 3.8% sodium citrate. The packed erythrocytes were obtained by centrifugation at 1000 × g for 10 min at 4°C. The plasma, separated by aspiration was used for vitamin C and E assay. The buffy coat was discarded and the erythrocytes were washed thrice in normal saline. Hemolysates were then prepared according to the method of Goldberg and Boches [19]. The washed packed erythrocytes were lysed in 5–10 times their volume of deionized water; 20 mM PMSF was added to the packed cells to be used later for the assay of the antioxidant enzymes. The addition of PMSF did not affect the enzyme activity under our experimental conditions.

In another set of preparations, after lysis of the packed erythrocytes in deionized water, the suspension was centrifuged at 100,000 × g for 1 h at 4°C. The supernatants were then stored at –20°C to be

used in the future for the measurement of proteolytic activity and the assay of the antioxidant enzymes *viz*. catalase, glutathione peroxidase (GSH-Px) and GR.

Removal of contaminating hemoglobin from the hemolysate

To extract Cu-Zn SOD, the hemoglobin present in the hemolysate was removed according to the method of McCord and Fridovich [30]. Briefly, 2.5 ml of the hemolysate was prewarmed at 37°C and treated with 1 ml of ethanol-chloroform (2:1, v/v) and mixed thoroughly to obtain a thick precipitate. Two milliliters of deionized water was added, mixed again and incubated at 37°C for 15 min with occasional stirring. The mixture was then centrifuged to spin down the precipitate. The colorless supernatant, thus obtained, was diluted with deionized water in a ratio of 1:1. This hemoglobin-free preparation was then used for superoxide dismutase (SOD) assay.

Preparation of erythrocyte membrane

Hb-free erythrocyte membrane preparation (either normal or treated) was prepared according to method of Arduini et al. [2]. The washed erythrocytes were subjected to hypotonic lysis in 40 volumes of 5 mM sodium phosphate buffer (pH 8.0) and centrifuged at 6000 × g for 20 min at 4°C. The supernatant was discarded and pellet was washed at least five times in the same buffer until a colorless pellet was obtained. The erythrocyte ghosts were suspended in the same buffer and stored at –20°C for future use. The protein concentration in the erythrocyte suspensions, membranes and lysate was determined according to the method of Bradford [6].

Measurement of protein degradation

The proteolytic activity in the thalassemia patients was studied by incubating the membrane protein (1.5 mg/ml of treated membrane) with erythrocyte lysate (0.15 mg/ml) in a final volume of 1 ml for 1 h at room temperature. The resulting tyrosine release was measured by the method of Waalkes and Udenfriend [45]. Briefly, after the incubation, the reaction was stopped by the addition of 1 ml of 30% trichloroacetic acid (TCA). A 1.5 ml of the above acid-soluble supernatant was further incubated with 1 ml of 0.1% of nitrosonaphthol and 1ml of nitric acid reagent in a total volume of 3.5 ml. The incubation was carried out in a water-bath at 55°C for 30 min. Unreacted nitrosonaphthol was extracted with 5 ml of ethylene dichloride followed by centrifugation, and the fluorescence of tyrosine derivative was measured from the supernatant (aqueous layer) using 460 nm as an excitation and 570 nm as an emission wavelengths using a Hitachi spectrofluorometer.

SDS-PAGE analysis

Electrophoresis was carried out according to the method of Laemmli [26] using a 10% polyacrylamide gel. Both treated and untreated membrane preparations as well as thalassemic erythrocyte membrane preparations containing the same amount of protein were solubilized by boiling for 5 min in SDS sample buffer containing 1% SDS, 10% (v/v) glycerol, 63 mM Tris-HCl (pH 6.5) and 1% β-mercaptoethanol. Equal amounts of membrane proteins (50 μg) were loaded on the gel. Gels were fixed and stained with 0.1% Coomassie blue R-250.

Determination of lipid peroxidation

The level of thiobarbituric acid reactive substances (TBARS) was measured as an index of peroxidation of lipids in the erythrocytes using the method of Beuge and Aust [7]. Two ml of TBA-TCA-HCl reagent was added to 1 ml of a 20% suspension of washed erythrocytes. The mixture was boiled in a boiling water-bath for 15 min. After centrifugation, the absorbance of the supernatant was recorded spectrophotometrically at 532 nm. A blank consisting of 1 ml of saline and 2 ml of TBA-TCA-HCl reagent was always carried out and any absorbance due to reagents was subtracted from the corresponding experimental sample. The TBARS content in the sample was calculated using an extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$.

Assay of antioxidant enzymes

Catalase

The erythrocyte catalase activity was measured following the rate of decomposition of H_2O_2 by the method of Beers and Sizer [5]. The hemolysate was diluted at the ratio of 1:10 with saline. Two milliliters of 50 mM phosphate buffer (pH 7.0) and 0.01 ml of the diluted hemolysate (enzyme) was added into the cuvette. At zero time, 1 ml of 5×10^{-3}M solution of buffered H_2O_2 was added into the cuvette. The contents were mixed quickly and the changes in ab-

sorbance were recorded at 240 nm every 10 s. The specific activity was expressed as micromoles of H_2O_2 consumed within one minute.

Superoxide dismutase

Superoxide dismutase was assayed according to the method of Paoletti and Mocali [34], based on NADPH oxidation. The method consists of a purely chemical reaction sequence which generates superoxide anion from molecular oxygen in the presence of EDTA, manganese (II) chloride and mercaptoethanol. The reaction mixture in a final volume of 2 ml contained 1.5 ml of 100 mM triethanolamine-diethanolamine-HCl buffer (TDB), 80 µl of 7.5 mM NADPH, 50 µl of 100 mM EDTA-$MnCl_2$ and 0.2 ml of hemolysate (sample solvent for control). The contents were mixed and allowed to stand for 5 min for a stable baseline. The reaction was initiated by the addition of 0.2 ml of 10 mM mercaptoethanol. The contents of the cuvette were mixed and the decrease in absorbance at 340 nm was followed for 10 min to allow NADPH oxidation.

Glutathione peroxidase

GSH-Px was carried out according to the method of Paglia and Valentine [33]. In this method GSH-Px catalyzes the reduction of H_2O_2 in the presence of reduced glutathione. The assay system contained 0.43 M phosphate buffer containing 2 mM EDTA (pH 7.0), 0.025 mM sodium azide, 0.15 mM glutathione, 1 unit of GR, 10 µl of undiluted hydrolysate and 0.25 mM of NADPH. The reaction was initiated by the addition of 0.36 mM H_2O_2. The linear decrease in NADPH absorption was recorded at 340 nm. The specific activity of the enzyme was calculated as micromoles of NADP produced within a minute per mg of protein.

Glutathione reductase

GR assay was carried out according to the method of Krohne-Ehrich et al. [25]. The assay mixture in final volume of 3 ml contained 50 mM phosphate buffer, 200 mM KCl, 1 mM EDTA and water. The blank was set with this mixture. Then, 0.1 mM NADPH was added together with 20 µl of undiluted hemolysate (enzyme) into the cuvette. The reaction was initiated with 1 mM oxidized glutathione (GSSG). Here also, the decrease in NADPH absorption was monitored at 340 nm. The specific activity of the enzyme was calculated as µmols of NADP produced/min/mg protein.

Assay of ascorbic acid (vitamin C)

Determination of ascorbic acid was carried out by the method of Omaye et al. [31], which involves a reduction of 2,6-dichlorophenolindophenol (DCIP). Ascorbic acid reduces the dye DCIP and causes a decrease in the absorption of the dye at 520 nm. The reaction was carried out within a pH range of 3.0–4.5.

Briefly, the procedure includes addition of 2 ml of 5% TCA to 1 ml of plasma to deproteinize it followed by centrifugation at 14,000 × g for 20 min at 4°C. The supernatant thus obtained was stored at –70°C for further analysis. For asorbate assay, 0.61 ml of the thawed supernatant was mixed with 0.33 ml of citrate/acetate buffer (pH 4.15). To this, 0.33 ml of DCIP was added and the absorbance was recorded against deionized water at 520 nm exactly after 30 s. A few crystals of ascorbic acid were added to bleach the dye by reducing it completely, and, the sample was read again. This value serves as the blank for the same sample. A standard curve, including a reagent blank, was constructed with the standards ranging between 0 and 20 µg of ascorbic acid per ml of 5% TCA. A change in absorbance (A) due to reduction of the dye by ascorbic acid in the sample was calculated from the following equation:

$$\Delta A = (RB - RB_b) - (S - S_b)$$

where RB is the absorbance of the reagent blank; RB_b is the absorbance of RB after bleaching with ascorbic acid; S is the absorbance of the sample and S_b is the absorbance of S after bleaching with ascorbic acid. The concentration of the ascorbic acid in the sample was obtained by comparing ΔA with the standard curve and expressed as µg/ml of plasma.

Assay of total tocopherol (vitamin E) in plasma

Plasma total tocopherol was assayed by the method of Quaife et al. [37]. It involves the Emmerie-Engel color reaction with ferric chloride and α, α'-dipyridyl to give a red color. Plasma was collected and stored at –20°C. The 0.6 ml of absolute ethanol was added to 0.6 ml of thawed plasma and vortexed well. Thereafter, 0.6 ml of xylene was added to this mixture, mixed well and centrifuged for 10 min at 3000 rpm. The 0.4 ml of the xylene supernatant layer was transferred to another tube and 0.4 ml of α, α'-dipyridyl reagent was added

and vortexed. The 0.6 ml of this mixture was then pipetted into a cuvette and the absorption was measured spectrophotometrically at 460 nm against deionized water. A 0.13 ml of ferric chloride reagent was then added to the cuvette, mixed thoroughly and absorption was again read at 520 nm spectrophotometrically exactly 1.5 min after addition of ferric chloride.

A calibration curve was prepared from the results obtained on solutions in absolute ethanol ranging in concentrations from 0.5 to 2.0 mg per 100 ml. A 0.6 ml of these were added to equal volume of deionized water and treated as described for plasma. When all values of the standard and unknown are corrected for reagent blanks, the calculation for vitamin E (total free tocopherol) in plasma becomes:

$$\text{Mg\% vitamin E} = D_{520} - 0.29\ D_{460}/D_{520} \times \text{mg\% vitamin E in the standard sample.}$$

Estimation of α-tocopherol in erythrocytes

α-Tocopherol was estimated by the fluorometric method of Chiu and Lubin [13]. After removal of plasma and the buffy coat, the erythrocytes were washed twice with 5 mM phosphate-buffered saline (PBS) (pH 7.4) and stored at –20°C for no more than four weeks prior to vitamin E estimation. A mixture consisting of 2 ml of 5 mM PBS (pH 7.4), 0.5 ml of 25% ascorbic acid, 1 ml of absolute ethanol and 0.25 ml of washed erythrocytes was prepared. This was incubated at 70°C for 5 min. Then 1 ml of 10 M KOH was added and the mixture was saponified further for 30 min at 70°C. After cooling, the saponified mixture was extracted with 4 ml of hexane for 1 min with vortexing. The extracted mixture was centrifuged and a portion of the hexane phase was removed for the determination of vitamin E content fluorometrically using 290 nm as an excitation and 350 nm as emission wavelength. The erythrocyte vitamin E was expressed as μg of vitamin E/ml of erythrocytes. For blank, water was substituted for erythrocytes and a standard was prepared by the addition of 1 ml of ethanol to mixture that contained 2.5 μg of α-tocopherol in 1.5 ml of water and treated further as erythrocytes. α-Tocopherol concentrations were calculated from comparison of the sample fluorescence intensity with that of standard after correction had been made for blank.

Statistical analysis

Data are presented as means ± SEM. Student's *t*-test was used for determination of the level of significance.

RESULTS

The level of Hb, plasma vitamin C and E, erythrocyte vitamin E content as well as lipid peroxidation in the erythrocyte membrane are shown in Table 1 for normal subjects, and for vitamin E-treated as well as untreated Eβ-thalassemic and β-thalassemic patients. The table reveals that the Hb level is significantly lower in both Eβ-, and β-thalassemic patients when compared to otherwise normal subjects. Although patients suffering from Eβ-thalassemia did not show any improvement, the Hb level in β-thalassemic patients improved significantly (28.2% over untreated subjects) after four weeks of vitamin E treatment when compared to the level in the untreated patients. However, in both types of thalassemic patients the Hb level, even after vitamin E treatment, never reach the values (13.75 g/dl) observed in normal subjects. Table 1 further reveals that plasma vitamin C level in both Eβ- and β-thalassemic patients remains significantly reduced (by more than 63% and 70% in Eβ- and β-thalassemic patients, respectively, compared to normal subjects) and treatment of the patients with vitamin E showed significant improvement in plasma ascorbate though not reaching ascorbate level observed in normal subjects. There is not much difference observed in case of plasma vitamin E level in normal subjects and the Eβ- and β-thalassemic patients. However, after vitamin E therapy, patients with Eβ-thalassemia exhibited a significant improvement in the plasma level of vitamin E when compared to the level in untreated Eβ-thalassemic patients. The plasma vitamin E content of β-thalassemic patients did not show any improvement after four weeks of vitamin E treatment. The erythrocyte vitamin E content also remained depressed in both types of thalassemic patients when compared to normal subjects, and the patients with Eβ-thalassemia again were those who showed a significant improvement in the erythrocyte vitamin E content (by 59% over the untreated group) when compared to the Eβ-thalassemic patients not treated with vitamin E for the same period of time. Erythrocyte membranes from both the

Table 1. Effects of vitamin E treatment on the hemoglobin level, vitamin C and vitamin E content in plasma, vitamin E in RBC and, lipid peroxidation in transfusion- dependent Eβ- and β-thalassemic patients

Parameters	Normal (N = 6)	Eβ-thalassemia (N = 9)		β-thalassemia (N = 8)	
		Before treatment	After 4 weeks of vitamin E treatment	Before treatment	After 4 weeks of vitamin E treatment
Pretransfusion hemoglobin level (g/dl)	13.75 ± 1.5	7.53 ± 0.94 ($p < 0.001$)	8.74 ± 1.12[NS2]	7.86 ± 0.58* ($p < 0.001$)	10.94 ± 1.38** ($p < 0.01$)
Vitamin C level in plasma (μg/ml)	10.3 ± 1.03	3.74 ± 0.46* ($p < 0.001$)	7.56 ± 0.83** ($p < 0.001$)	3.06 ± 0.19 ($p < 0.001$)	5.2 ± 1.1** ($p < 0.05$)
Vitamin E level in plasma (mg%)	1.74 ± 0.16	1.18 ± 0.19* ($p < 0.01$)	2.18 ± 0.16** ($p < 0.001$)	1.25 ± 0.66[NS1]	1.25 ± 0.60[NS2]
Vitamin E in RBC (μg/ml RBC)	1.92 ± 0.34	0.75 ± 0.18* ($p < 0.001$)	1.83 ± 0.15** ($p < 0.001$)	0.59 ± 0.11* ($p < 0.001$)	0.60 ± 0.15[NS2]
Lipid peroxidation (nmols TBARS/ mg of protein)	0.229 ± 0.0	0.362 ± 0.13* ($p < 0.01$)	0.245 ± 0.08** ($p < 0.001$)	0.446 ± 0.14* ($p < 0.001$)	0.289 ± 0.09** ($p < 0.05$)

Values are means ± SEM, *p *vs*. normal, ** *vs*. before treatment value, NS[1] (not significant) *vs*. normal, NS[2] (not significant) *vs*. before treatment values

Table 2. Activities of catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase in normal subjects and, transfusion-dependent Eβ- and β-thalassemic before and after vitamin E treatment

Parameters	Normal (N = 8)	Eβ-Thalassemia (N = 9)		β-Thalassemia (N = 9)	
		Before treatment	After 4 weeks of treatment	Before treatment	After 4 weeks of treatment
Catalase (μM H_2O_2 consumed/min)	8.64 ± 0.59	7.09 ± 0.65* ($p < 0.01$)	14.5 ± 0.52** ($p < 0.001$)	6.7 ± 0.7** ($p < 0.001$)	10.0 ± 0.68**[1]
Superoxide dismutase (Units/mg protein)	51.49 ± 4.5	112.96 ± 10.32* ($p < 0.001$)	50.00 ± 6.00** ($p < 0.001$)	72.32 ± 8.00* ($p < 0.001$)	52.55 ± 7.75**[2] ($p < 0.001$)
Glutathione peroxidase (mol NADP produced/ min/mg protein)	1.74 ± 0.04	0.73 ± 0.042* ($p < 0.001$)	1.8 ± 0.035** ($p < 0.001$)	0.69 ± 0.06* ($p < 0.001$)	1.66 ± 0.05**[3]
Glutathione reductase (mol NADP produced/ min/mg protein)	0.06 ± 0.003	0.028 ± 0.001* ($p < 0.001$)	0.03 ± 0.001[NS1]	0.034 ± 0.002* ($p < 0.001$)	0.04 ± 0.015[NS2]

Values are mean ± SEM, *p vs. normal, **p vs. values before treatment in the Eβ-thalassemic, **[1]p; **[2]p; **[3]p vs. respective values before treatment in the β–thalassemic, [NS1]; [NS2]= not significant when compared to the respective values obtained before treatment

Eβ- and β-thalassemic patients showed high level of lipid peroxidation when compared to normal subjects (more than 36.7% and 48% in Eβ- and β-thalassemic, respectively, over the normal subjects) and treatment of the patients with vitamin E for a period of four weeks remarkably reduced the level of lipid peroxidation in erythrocyte membranes.

The status of the antioxidant enzymes in the erythrocytes of normal subjects, Eβ- as well as β-thalassemic patients before and after vitamin E

Table 3. Protein degradation in transfusion-dependent Eβ- and β–thalassemic patients

Groups	Parameter
	Protein degradation (tyrosine release [µg/ml/h]
Normal (N = 9)	2.06 ± 0.64
Eβ-thalassemia (n = 9)	7.69 ± 0.73* ($p < 0.001$)
β-thalassemia (N = 9)	12.78 ± 0.92* ($p < 0.001$)

Values are means ± SEM, *p *vs*. normal

treatment was also studied and the results are presented in Table 2. The erythrocyte catalase activity was found to be significantly decreased in Eβ- and β-thalassemic patients with the degree of inhibition slightly higher (22.5%) in β-thalassemic patients compared to normal subjects. A significant improvement (twofold in Eβ-thalassemic and more than 66% in β-thalassemic) in catalase activity was evident after treatment of the patients with vitamin E for four weeks. The activity of erythrocyte Cu-Zn SOD of the Eβ- and β-thalassemic patients was significantly higher than normal subjects. The Cu-Zn SOD activity was restored to normal values when the patients were treated with vitamin E. Decreased catalase activity with an accompanying increase in the SOD activity is clearly indicative of an oxidative stress and enhanced generation of reactive oxygen species in the thalassemic erythrocytes. Further, the activities of GSH-Px and GR of normal and Eβ- and β-thalassemic patients were also studied (Tab. 2). GSH-Px activity was found to be decreased in both the types of thalassemia and activity returned to almost normal after therapy with vitamin E. GR, another important antioxidant enzyme, remained depressed in both the types of thalassemia when compared to normal subjects but the activity did not return to normal on treatment of the patients with vitamin E for a period of four weeks.

That the thalassemic patients are under constant oxidative stress is evident from the increased proteolytic activity measured by the release of tyrosine in the incubation mixture containing the membrane prepared from PHX-treated erythrocytes (which is a better substrate than normal erythrocyte membrane [15]) and the lysate isolated from the thalassemic erythrocytes. A tremendous increase in the degradation of protein (measured in terms of tyrosine release) was detected in both Eβ- and β-thalassemic patients when compared to normal subjects (Tab. 3). Furthermore, the SDS-PAGE analysis (Fig. 1) of the membrane prepared from thalassemic erythrocytes confirmed the above results. Most of the bands on the gel corresponding to different membrane proteins showed varying degrees of decrease in intensity. Spectrin was decreased slightly with the bands 2.1, 3 and actin showing a marked decrease thereby indicating a correlation between increased lipid peroxidation, proteolytic activity and degradation of cytoskeletal proteins. On the other hand, bands 4.1 and 4.2 (Fig. 1, lanes 1 and 2) showed a considerable decrease in their susceptibility to degradation under oxidative threat.

SDS-PAGE analysis of the erythrocyte membrane prepared from the blood of thalassemic patients before and after vitamin E treatment for three months further revealed that this lipophilic antioxidant was beneficial to thalassemic erythrocytes. Distinct changes are evident in the erythrocyte membrane protein profiles after vitamin E treatment. Bands 2.1, 2.2 and 4 are distinctly visible in lanes 2 and 4 in Figure 2 in comparison to lanes 1 and 3 representing membrane protein profiles from the same patients before the treatment with vitamin E. Finally, thalassemic patients also exhibited a distinct change in their body weight after vitamin E treatment for a period of three months. The body weight of both Eβ- and β-thalassemic patients increased in the range 3% to 20% as shown in the

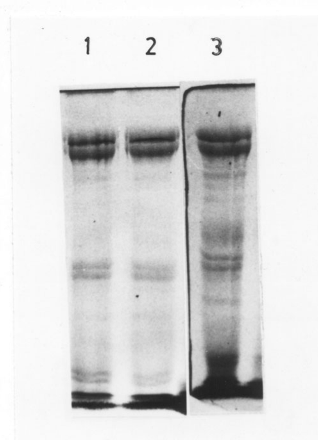

Fig. 1. SDS-PAGE of red cell membrane protein. Identical amounts of protein were applied to each lane (50 µg). Lane 1 – Eβ-thalassemia, Lane 2 – β-thalassemia, Lane 3 – Normal. Spectrin, band 2.1, band 3, band 4.1, band 4.2 and actin are the major erythrocyte cytoskeletal proteins to be affected by oxidative threat

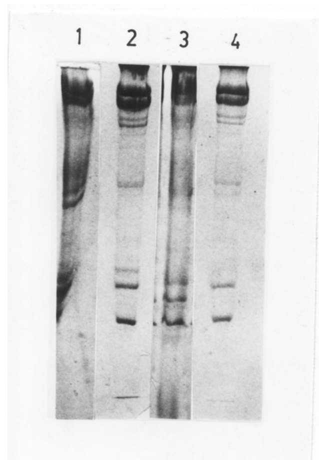

Fig. 2. SDS-PAGE of erythrocyte membrane protein before and after treatment of the thalassemic patient with vitamin E. Equal amounts of protein (50g) were applied to each lane. Lane 1 – Eβ-thalassemia before vitamin E treatment, Lane 2 – Eβ-thalassemia after vitamin E treatment, Lane 3 – β-thalassemia before vitamin E treatment, Lane 4 – β-thalassemia after vitamin E treatment. Band 2.1, band 2.2, and band 4 of the erythrocyte cytoskeletal membrane proteins showed improvement in their organization after vitamin E treatment. This is a representative result of three separate analyses

Table 4. Changes in body weights (in kg) after vitamin E treatment in transfusion-dependent Eβ- and β-thalassemic patients

Eβ-thalassemia			β-thalassemia		
Before treatment	After 12 week treatment	Increase %	Before treatment	After 12 week treatment	Increase %
16.0	17.0	6.25	10.0	12.0	20.0
43.0	45.0	4.65	22.0	23.0	4.5
21.0	22.5	7.14	15.0	16.0	6.6
18.5	19.5	5.41	12.5	13.5	8.0
46.0	48.5	5.43	9.0	9.5	5.6

Table 4. Further, as per an oral questionnaire, the patients reported for generally feeling good (reports not shown) indicating that vitamin E treatment may have an overall health promoting effect apart from having its specific effects on the erythrocytes.

DISCUSSION

The salient points of the present studies are:

1. The erythrocytes of the patients suffering from Eβ- and β-thalassemia remain under severe oxidative stress as is evident from the reduced level of cellular enzymatic and non-enzymatic antioxidant(s), increased level of membrane lipid peroxidation, as well as protein degradation.

2. Treatment of the patients with vitamin E for a period of 4 weeks restored the changes observed in the erythrocytes of the Eβ- and β-thalassemics to near normal indicating that administration of this natural lipophilic antioxidant may have the potential to extend the otherwise reduced longevity of the thalassemic erythrocytes and maintenance of the general well-being of the thalassemics.

Normal erythrocytes are also exposed to continuous oxidative stress but they generally show little evidence of a cumulative oxidant-mediated damage due to very effective enzymatic as well as non-enzymatic antioxidant defense system directed against a collage of oxidants [21]. The normal erythrocyte has been shown to have a reducing capacity which is greater 250 times than its oxidizing potential [42]. However, in thalassemic erythrocytes the antioxidant system seems to be insufficient [10, 44]. Catalase, GSH-Px and GR were found, in the current studies, to exhibit reduced activities when compared to the values obtained from the erythrocytes of the normal subjects (Tab. 2). On the other hand, SOD activity was found to be very high in both Eβ- and β-thalassemia when compared with normal erythrocytes (Tab. 2). A decrease in catalase activity with a concomitant increase in SOD activity is indicative of a situation in which there remains a fairly high possibility of accumulation of intracellular H_2O_2 which, in the presence of redox active transition metals like Cu^{2+} and Fe^{3+}, may be converted to the hydroxyl radical ($OH^•$) – the most dreaded of the ROS [14, 12]. The $OH^•$ is capable of reacting with anything in its vicinity with diffusion-controlled rate to cause oxidative damage of membrane lipids, proteins and cellular enzymes [4, 20]. The decreased catalase and increased SOD activities with consequential oxidative damage due to accumulating ROS has also been demonstrated in other models of oxidative stress [3].

The metabolism of the important cellular antioxidant, *viz*. reduced glutathione, seems to be affected in the erythrocytes of both types of thalassemics indicating the erythrocytes to be in a pro-oxidant state [10]. The present investigations additionally reveal a significant reduction in the activities of glutathione peroxidase and glutathione reductase in both Eβ- and β-thalassemia when

compared to erythrocytes from normal subjects. Therefore, a reduction in cellular antioxidants like reduced glutathione (as reported by earlier workers [10]), ascorbate and vitamin E as well as changes in the activities of the antioxidant enzymes (as currently being reported) (Tabs. 1 and 2) are clearly indicative of a severe oxidative stress in the erythrocytes of Eβ- and β-thalassemic patients.

The chemistry and biology of vitamin E has been the subject of intensive study for more than 50 years [16, 18, 28, 29, 32, 40], and enormous body of literature demonstrates conclusively that the principal role of vitamin E is to protect tissue against unwanted, destructive oxidation [8, 35]. Although additional functions of vitamin E have been studied by various investigators [9, 22, 39, 43], the antioxidant function of vitamin E remains the most well-established [36]. In fact, vitamin E is the most effective lipid soluble antioxidant present in our cells [36]. Interestingly, treatment of the thalassemic patients with vitamin E for a period of four weeks restored most of the enzymatic antioxidants of the erythrocytes to values near normal. However, the reason for the activity of GR not getting restored to the normal values even after four weeks of vitamin E treatment needs further investigation. In a different study [44], an oral treatment with vitamin E of β-thalassemia intermedia patients, not requiring chronic transfusional therapy, improves the antioxidant/oxidant balance in plasma, LDL particles and also counteracts lipid peroxidation processes in erythrocytes. However, the dose of vitamin E employed for that study was considerably higher and the period of treatment longer. In contrast, we have used a low dose of vitamin E for only four weeks with striking improvements in the transfusion-dependant Eβ- and β-thalassemic patients.

However the level of vitamin C and E in plasma as well as of vitamin E in thalassemic erythrocytes increased after treatment of the patients with vitamin E, the level of these antioxidant vitamins never reached the values found in the normal individual. This could either be due to a faulty absorption of these vitamins from the gastrointestinal tract or a considerable amount of these vitamin antioxidants still consumed to scavenge the reactive intermediate(s) generated due to oxidative stress. However, other possibilities may not also be ruled out.

That the erythrocytes of the Eβ- and β-thalassemic patients are under increased oxidative stress is also evident from an increased level of membrane lipid peroxidation as observed in our studies. However, vitamin E treatment reduces the burden of membrane TBARS indicating that vitamin E could be of potential use in the thalassemic patients to reduce the oxidant burden in erythrocyte and blood as a whole.

Continued oxidative stress in the thalassemic erythrocyte leads to oxidative modification and breakdown of several membrane proteins. This is also reflected in an increase in tyrosine release as an indicator of protein oxidation and degradation in both Eβ- and β-thalassemic patients (Tab. 3). This observation is further supported by SDS-PAGE analysis of the erythrocyte membrane proteins from the normal subjects and, Eβ- and β-thalassemic patients (Fig. 1) which clearly shows alterations in a number of important membrane cytoskeletal proteins when compared to normal membrane proteins from normal erythrocytes. SDS -PAGE analysis of erythrocyte membrane proteins of patients undergoing vitamin E treatment (Fig. 2) further shows a striking improvement in the intensity of erythrocyte cytoskeletal membrane protein bands with a reduction of low molecular weight protein aggregates. Furthermore, some aggregated polypeptides can be seen at the top of the gel in the Figure 2 that decreases after vitamin E treatment. Understandably, the protective role of vitamin E may lie in the fact that it reduces the propagation of lipid peroxidation and accumulation of TBARS by scavenging the reactive intermediates thereby reducing protein degradation and, thus, stabilizing the erythrocyte membrane proteins. The present studies further reveal that treatment of Eβ- and β-thalassemic patients with vitamin E, apart from protecting the erythrocytes, appears to maintain the general well-being of the patients (as reported by the patients according to a questionnaire) as reflected in the body weight gain (Tab. 4) after eight weeks of treatment.

The essence of the current studies lies in the fact that, perhaps, this is the first study to demonstrate and compare the oxidative stress status in the erythrocytes of normal subjects as well as Eβ- and β-thalassemic patients. Further, this work also provides evidence for the ability of the lipophilic antioxidant vitamin E to protect the ailing erythrocytes of the transfusion-dependent thalassemic patients from undergoing premature lysis in the face of continuing oxidative stress.

Acknowledgments. We are greatly indebted to Dr. A. Sarkar of Indian Association of Blood Cancer and Allied Disease (IABCD), Calcutta, and his staff for clinically controlling the vitamin E study and helping in the collection of blood and management of patient data. We also thank the staff of Ramakrishna Mission Seva Pratisthan (Thalassemia Unit), Calcutta, and the staff of Hemophilia Society of India (Calcutta chapter) for providing us with patient's blood and also drawing normal blood samples for us.

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Received: *May 26, 2003; in revised form: November 19, 2003.*