

*Original Article***The effects of beta-carotene and vitamin E on erythrocytes lipid peroxidation in beta-thalassemia patients**

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Abstract

BACKGROUND: Thalassemia is the most common hereditary disease in the world. Thalassemic erythrocytes are exposed to higher oxidative stress and lipid peroxidation. The aim of this study was to investigate the effects of beta-carotene and vitamin E on erythrocytes lipid peroxidation in beta-thalassemia patients.

METHODS: A prospective double-blind, placebo-controlled study of the effect of beta-carotene and vitamin E on lipid peroxidation in erythrocytes membranes was performed on 120 beta-thalassemia major patients in four groups. The patients were supplemented for 4 weeks as follows: group 1 with beta-carotene (13 mg/day), group 2 with vitamin E (550 mg/day), group 3 with beta-carotene plus vitamin E and group 4 with placebo. We prepared all capsules for 4 groups in the same shape and color. Measurements of serum beta-carotene and vitamin E were performed by high performance liquid chromatography. After preparation of ghost cells from blood specimens, malondialdehyde (MDA) was determined as index of lipid peroxidation in erythrocytes membranes before and after treatment.

RESULTS: The levels of serum beta-carotene and vitamin E were significantly lower and MDA concentrations in erythrocytes membranes were significantly higher in beta-thalassemia patients compared to controls ($P < 0.001$). In groups that treated with vitamin supplements for 4-weeks, lipid peroxidation rates were significantly reduced after treatment ($P < 0.001$), but in placebo group there was not significant difference ($P > 0.05$).

CONCLUSIONS: Our findings provide evidence that an oral treatment with beta-carotene and vitamin E can significantly reduce lipid peroxidation of erythrocytes membranes and could be useful in management of beta-thalassemia major patients.

KEYWORDS: Beta-thalassemia major, beta-carotene, vitamin E, malondialdehyde, lipid peroxidation.

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Thalassemia is the most common hereditary disease in the world and is a paradigm of monogenic genetic diseases¹. Thalassemia major is the severe form of the disease, presenting with transfusion dependent anemia, generally in the first year of life². The abnormalities of erythrocytes of thalassemia result from the accumulation of the

unmatched normal globin chains that are present in excess³. 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

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and globin moieties, loading the erythrocyte membrane with denatured globin chains, heme and iron^{4,5}. Thalassemic erythrocytes are exposed to higher oxidative stress and a possible consequential accelerated apoptosis contributing to shortened life span, as a result of excess production of reactive oxygen intermediates within the erythrocytes⁶⁻⁸. 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^{9,10}. A cross-linked erythrocyte membrane is expected to be rigid and this could probably explain why the rigidity of thalassemic erythrocyte deformability is a major determinant of anemia in thalassemia^{11,12}. Very low and sometimes undetectable levels of serum vitamin E have been found in β -thalassemic major also known as Cooley's anemia^{13,14}. Low serum vitamin E levels have been found in several conditions associated with chronic steatorrhea, such as cystic fibrosis¹⁵, but this does not seem to be the case in thalassemia where there is no evidence in favor of malabsorption and particularly in lipid malabsorption as shown by the normal levels of β -carotene in the presence of severe vitamin E deficiency¹⁶ and by normal absorption of triglycerides, retinol, glucose and D-xylose¹⁷. Alternatively, and in view of the data indicating lipid membrane peroxidation in red blood cells of patients with β -thalassemia major, it has been postulated that the low serum vitamin E levels may be due to a secondary consumption of the antioxidant consequent to the membrane oxidation rather than a primary cause in its metabolism and absorption^{16,17}. The ultimate conclusion of these observations justified a therapeutic trial with vitamin E and β -carotene in patients with β -thalassemia major, aiming at neutralizing the deleterious oxidative damage of the red blood cells (RBC) membrane and eventual beneficial effects on RBC survival, the severity of the anemia and

the transfusion requirements¹⁸⁻²¹. The present report describes the results of such a therapeutic trial with its effects on erythrocytes lipid peroxidation in patients with β -thalassemia major.

Methods

Materials: Trichloroacetic acid (TCA), thiobarbituric acid (TBA), hydrochloric acid (HCL), reagents of high performance liquid chromatography (HPLC) grade as methanol, ethanol, hexane, acetonitrile, tetrahydrofuran, diethyl ether were purchased from Merck Company, Darmstadt, Germany; α -tocopherol standard and 1,1,3,3-tetraethoxypropane from Sigma Chemical Company, St Louis, MO, USA. β -carotene was purchased from Fluka Company. Other reagents used were of analytical grade.

Subjects and clinical samples: A prospective double-blind, placebo-controlled study of the effects of vitamin E and beta-carotene on lipid peroxidation in erythrocytes membranes was performed on 120 beta-thalassemia patients. Present study was conducted in Thalassemia Center of Children Amirkola Hospital and Departments of Biochemistry and Pharmacology in Babol University of Medical Sciences, Babol, Iran. All patients were recruited, with informed consent and have been previously characterized for beta globin gene mutations. They were divided into 4 groups (matched with age and gender, n = 30). Nutritional questionnaire was filled out for each patient. Patients were treated for 4 weeks as follows: group 1 with beta carotene (13 mg/day), group 2 with vitamin E (550 mg/day), group 3 with beta-carotene plus vitamin E and group 4 with placebo. We prepared 4 kinds of capsules for 4 groups in the same shape and color. Blood from patients was collected just before the transfusion in two different tubes (EDTA and without anticoagulant). After supplement therapy for 4 weeks, blood was taken on 29th day of the experiment. Control population involved 30 healthy individuals age and gender matched with the patients population. Serum was separated after clotting by centrifugation

at $1000 \times g$ for 10 minutes and plasma was prepared by centrifugation of the blood sample at $3000 \times g$ for 15 minutes at 4°C . After separation of the buffy coat, the packed RBCs were washed 4 times with isotonic saline solution at pH 7.0.

Preparation of erythrocyte ghost membranes: Hb-free erythrocyte membrane preparation was prepared according to the method of Arduini et al.²² The washed erythrocytes were subjected to hypotonic lysis in 40 volumes of 5 mM sodium phosphate buffer (PH = 8.0) and centrifuged at $6000 \times g$ for 20 minutes at 4°C in a refrigerated centrifuge. 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.

Measurement of beta-carotene and vitamin E by HPLC: Beta-carotene and vitamin E were measured by HPLC as described by Driskell et al. with some modifications²³. The HPLC system consisted of a 20 μl injection loop, an isocratic pump, an ultraviolet-visible spectrophotometric detector (730D) and a C18 reversed-phase column Novapak C18, 3.9×150 mm, 4 μm from Waters Company, USA. The mobile phase consisted of methanol/acetonitrile/tetrahydrofuran (50: 45: 5v/v/v) at a flow rate of 1.5 ml/minute.

Measurement of lipid peroxidation: The level of thiobarbituric acid reactive substance (TBRAS) was measured as an index of lipid peroxidation in the erythrocytes using the method of Buege and Aust²⁴. 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 minutes. After cooling and centrifugation at $1000 \times g$ for 10 minutes, the absorbance of the supernatant was determined at 532 nm using UV/VIS spectrophotometer (Jenway 6505, UK). A blank control 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 standard curve was prepared using serial concentrations of 1,1,3,3-tetraethoxypropane (Sigma, St. Louis, MO, USA). The malondialdehyde-thiobarbituric acid (MDA-TBA) adduct was shown at 532 nm and quantified by reference to a standard curve of 1, 1, 3, 3-tetraethoxypropane, submitted to the TBA colorimetric procedure²⁵.

Statistical analysis: All results are expressed as mean \pm standard deviation (SD). Comparison between controls and thalassemia patients was performed by student's t-test. Paired student t-test was used for determination of the level of significance before and after treatment in β -thalassemia patients groups. The criterion for significance was $P < 0.05$.

Results

Some demographic data of patients with β -thalassemia major and controls are listed in table 1. The level of vitamin E in serum of β -thalassemia major patients was significantly lower compared to controls (420.83 ± 169.71 vs. 1203.5 ± 422.56 $\mu\text{g}/\text{dl}$) ($P < 0.001$). Also, serum β -carotene level in β -thalassemia major patients was lower compared to control group (17.70 ± 12.64 vs. 35.13 ± 13.95 $\mu\text{g}/\text{dl}$) ($P < 0.001$). However, the mean MDA concentration in erythrocytes membranes of β -thalassemia major patients was significantly higher compared to controls (0.50 ± 0.15 vs. 0.17 ± 0.11 nmol/ml) ($P < 0.001$). Table 2 shows the effects of supplement therapy on lipid peroxidation of erythrocytes membranes in β -thalassemia major patients. The mean malondialdehyde concentration in erythrocytes membranes of the patients that treated with vitamin supplements for 4 weeks, showed a significant decrease when compared to the mean MDA concentration before treatment ($P < 0.001$). Decrease of erythrocyte lipid peroxidation in group 3 that treated with β -carotene plus vitamin E was more than that in groups 1 and 2. There was not any significant

Table 1. Demographic data of β -thalassemia major patients groups and controls.

	Controls (n=30)	TM (β -carotene) (n=30)	TM (vitamin E) (n=30)	TM (β -carotene + vitamin E) (n=30)	TM (Placebo) (n=30)
Boys	16	13	15	16	13
Girls	14	17	15	14	17
Mean age (years)	19.71	17.79	17.82	18.48	19.20

TM: β -thalassemia major patients

Table 2. Effects of β -carotene and vitamin E treatment on the erythrocytes lipid peroxidation in β -thalassemia major patients groups.

Patients groups	Erythrocyte lipid peroxidation (nmol MDA/ml)		
	Before treatment	After 4-week treatment	P value
1. TM (β -carotene) (n = 30)	0.38 \pm 0.10	0.32 \pm 0.09	P<0.001
2. TM (vitamin E) (n = 30)	0.34 \pm 0.09	0.25 \pm 0.09	P<0.001
3. TM (β -carotene + vitamin E)(n = 30)	0.38 \pm 0.09	0.23 \pm 0.08	P<0.001
4. TM (Placebo) (n = 30)	0.40 \pm 0.12	0.37 \pm 0.10	P>0.05

TM: β -thalassemia major patients. Results are expressed as mean \pm SD; P<0.05 was considered significant.

difference in the mean MDA concentration before and after treatment in the placebo group (P>0.05).

Discussion

In the present study, treatment of the β -thalassemia major patients with β -carotene and vitamin E for a period of 4 weeks significantly reduced malondialdehyde concentration as an index of lipid peroxidation in erythrocytes membranes. Previous studies have demonstrated a variety of morphological, biochemical, and metabolic disturbances of the thalassaemic red cell with shortened life span^{14,26,27}. There is extensive evidence of in vivo oxidative damage as well as enhanced sensitivity to exogenous oxidant stress in red cells of β -thalassemia²⁶. It has been postulated that the biochemical and metabolic changes of β -thalassaemic red blood cells (RBCs) are associated with a constant oxidative stress within the cells caused by precipitation of excess alpha-globin chains, iron decompartmentalization and release of free iron^{5,6,8,26}. 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 systems directed against a collage of oxidants²⁸. The normal erythrocyte has been shown to have a reducing capacity which 250 times is greater than its oxidizing potential²⁹. We observed higher amounts of MDA in erythrocytes membranes of transfusion-dependent β -thalassemia major patients compared to controls. This finding confirms the report of Giardini et al. about concentration of MDA in RBC of β -thalassemia patients³⁰. Malondialdehyde, which results from oxidation of polyunsaturated fatty acids (PUFAs), is also present in high amounts in organs of the reticuloendothelial system (RES) of thalassaemic patients³¹. MDA is known to cause cross-linking of membrane components, thus increasing RBC membrane rigidity and decreasing RBC deformability^{32,33}. Lipid peroxidation plays an important role in RBC removal by the RES. It has been shown to alter membrane phospholipids asymmetry, an important determinant of RBC recognition by

macrophages^{33,34}. Furthermore, RBCs treated in vitro with abnormal lipid distribution were phagocytosed 4 times more readily than control RBCs³⁵. In our study, the levels of β -carotene and vitamin E in serum of β -thalassemia major were significantly lower compared to control groups. Vitamin E is considered as pathophysiologically important determinant of antioxidative protection³⁶. In fact, vitamin E is the most effective lipid soluble antioxidant present in our cells³⁷. Vitamin E deficiency in β -thalassemia major patients is due to its increased consumption as a result of the oxidative stress^{17,27}. Simsek et al. found significant difference in serum vitamin E level between β -thalassemia major and control groups²⁷. Kassab-Chekir et al. demonstrated that vitamin E level in serum of β -thalassemia patients decreased by 70%. However, they did not find any significant difference concerning serum vitamin A between the patients and control groups³⁸. We observed significant reduction in MDA concentration of erythrocytes membranes in beta-thalassemia patients after treating with vitamin supplements for 4 weeks. This result is in agreement with Das et al. study²¹ that found significant decrease concerning lipid peroxidation in erythrocytes membranes of thalassemia patients after treatment with vitamin E. However, they did not use a therapeutic trial with β -carotene for thalassemic patients and subjects of each group were less than 10. In a different study, an oral treatment with vitamin E of β -thalassemia intermedia patients not requiring chronic transfusional therapy, improved the antioxidant/oxidant balance in plasma, LDL particles and also did counteract lipid peroxidation processes in erythrocytes¹⁹. However, the dose

of vitamin E employed for that study was considerably higher and the period of treatment longer compared to our study. We demonstrated that in group 3 treated with β -carotene plus vitamin E, reduction of MDA concentration was more than that in the two other groups (1 and 2) that treated with β -carotene or vitamin E alone. This may be due to synergistic effects. Presence of vitamin E and lipid soluble antioxidants in suitable amounts in the RBC membranes and their synergistic interactions, guarantee membrane structural integrity^{39,40}. Dissayabutra et al. found that supplement therapy with vitamin C plus vitamin E has more benefits compared with vitamin E alone concerning promoting antioxidant status in beta-thalassemia patients⁴¹. In conclusion, we believe that thalassemic RBCs, suffer from peroxidation damage to their membrane lipids as it can be shown by increased MDA levels and low levels of vitamin E. The described changes might contribute to the premature aging of these RBCs by generating "Senescent - cell antigen", which will ultimately lead to their removal from the circulation. The present study compared the lipid peroxidation status in erythrocytes membranes of normal subjects and β -thalassemic patients. Further, this work also provided evidence for the ability of the lipophilic antioxidants β -carotene and vitamin E to protect the ailing erythrocytes of the transfusion-dependent thalassemic patients from lipid peroxidation.

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