EFFECTS OF ANTIOXIDANT VITAMINS ON SOME HEMOGLOBIN PROPERTIES AND ERYTHROCYTES IN HOMOZYGOUS BETA-THALASSEMIA

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Abstract. The present work is concerned with the study of the effects of antioxidant vitamins on the erythrocytes, hemoglobin (Hb) derivatives and the rate of alkaline denaturation of Hb in homozygous β-thalassemic patients. The patients were treated with vitamins E, C and A for twelve months. The mean value of hemolysis of β-thalassemic children before treatment is significantly higher than that of controls, whereas, that of β-thalassemic children after treatment is significantly lower than that of β-thalassemic children before treatment. The results of multi-component spectrophotometric analysis revealed significant decreases in MetHb % and HbCO % concomitant with significant increases in HbO_2 % in β-thalassemic blood after treatment with antioxidant vitamins, when compared before treatment. The results of alkaline denaturation showed insignificant differences in the rate of this reaction between the normal Hb and beta thalassemic Hb before treatment and between anemic patients before and after treatment, respectively. The results of this method revealed values of HbF in the range (12.5–45.38%) and (9.289–33.99%) for β-thalassemic children before and after treatment with antioxidants, respectively. Finally, it seems clear that treatment of β-thalassemic patients with antioxidant vitamins reduced the hemolysis of red blood cells and hence improved the hemoglobin levels of thalassemic patients. Also, treatment with antioxidants vitamins reduced the levels of inactive Hb (HbCO and MetHb) and hence improved the active Hb (HbO_2) concentrations, whereas it had no effects on the rate of alkaline denaturation of Hb.

Key words: β-thalassemia, antioxidant vitamins, erythrocytes hemolysis, hemoglobin derivatives, multi-component spectrophotometric analysis, fetal hemoglobin, alkaline denaturation of hemoglobin.

INTRODUCTION

Thalassemia is a group of inherited hemoglobin disorders characterized by reduced synthesis of one or more of the globin chains leading to imbalanced globin synthesis which is the major factor in determining the severity of the disease in the thalassemia syndromes. In Egypt, β-thalassemia is the commonest cause of chronic

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hemolytic anemia and it represents a major genetic disease and a public health problem which engulfs a large portion of the country's health financial plan. But, before considering therapy, it is rather critical to confirm thalassemia major in patients and to eliminate concomitant causes of anemia [13, 26].

The fundamental problem in β-thalassemias is the uncoupling of α- and β-chain synthesis. Normally, these chains are made in almost equal quantities. In β-thalassemias, however, β-chain production is depressed moderately, in the heterozygous form (β-thalassemia minor or trait), and very severely in the homozygous state (β-thalassemia major) [6].

In β-thalassemia syndromes, decreased or impaired biosynthesis of beta-globlin leads to accumulation of unpaired alpha globin chains. Excess presence of the alpha-globin chains is the primary reason for the cellular oxidative damage in thalassemias [22]. And also iron overloading as a result of both high plasma iron and high intracellular nonhemoglobin iron in β-thalassemias leads to an enhanced generation of reactive oxygen species and oxidative stress [20]**.**

Vitamins A, E & C, carotene and glutathione (GSH) provide antioxidant defense by their ability to exist in reversible oxidized and reduced forms. The enzymes superoxide dismutase (SOD) detoxifies superoxide radical and catalase and glutathione peroxidase (GPX) which act on H_2O_2 and hydroperoxide respectively serve as the endogeneous antioxidants [4]. Due to increased consumption of low plasma levels of tocopherol, a chain breaking antioxidant may induce lipid peroxidation within the red blood cells and consequently hemolysis [14]. The degree of lipid peroxidation in the organism can be evaluated by malonyldialdehyde (MDA), which is the breakdown product of lipid peroxidation [10]. Antioxidants, which are working against the oxidative damage within the cell, consist of preventive and chain breaking mechanisms. Superoxide dismutase (SOD) is a preventive antioxidant whereas vitamin E is a chain breaking antioxidant [11].

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 β-thalassemic patients when compared to normal subjects. However, the activity of superoxide dismutase was found to be increased in untreated thalassemic patients when compared to normal individuals. An increase in superoxide dismutase and a decrease in catalase activity reflect the presence of a severe oxidative stress situation in the erythrocytes of the untreated transfusion dependent β-thalassemia patients. Changes in erythrocyte membrane protein pattern in untreated β-thalassemia patients when compared to normal erythrocyte further confirm the presence of continued oxidative stress in the ailing thalassemic erythrocytes [12]. It is clear that patients with β-thalassemia suffer from chronic oxidative stress and have an altered redox state characterized by gross depletion of antioxidant nutrients [2]. Most of the clinical events in patients with this disorder

were precipitated directly by severe antioxidant depletion resulting in inadequate protection. Therefore, systematic large scale clinical trials involving the supplementation of combined antioxidant nutrients may generate useful information from which antioxidant replacement therapy may be used as adjunct treatment for this disabling condition.

Thus the efficacy of antioxidant therapy especially treatment by vitamin E was evaluated in a previous study [12]. In this study no improvements in vitamins E and C concentrations was observed after treatment. This may be attributed to the low dose of vitamin E (10 mg/kg·day) used in this study and to the absence of any treatment with vitamin C. Vitamin C has short term effect on vitamin E concentration in healthy subjects [19]. However, the interaction between the two vitamins and their effects on the erythrocytes hemolysis and hemoglobin properties in β-thalassemic has not been studied yet**.** Moreover, the effects of vitamin A treatment on the erythrocytes and hemoglobin in β-thalassemics have not been evaluated. A recent study reported lower levels of vitamin A in homozygous β-thalassemias than in controls [31].

Therefore, we aimed to study the effects of antioxidant vitamins treatment on the erythrocytes hemolysis, Hb derivatives and the rate of alkaline denaturation of Hb in patients with homozygous β-thalassemia.

MATERIALS AND METHODS

PATIENTS AND BLOOD COLLECTION

The current study involved ten homozygous β-thalassemic patients aged 5–16 years (5 males and 5 females) admitted to Hematology and Oncology Department, Pediatric Hospital, Ain Shams University. A group of 8 children of the same age and sex and free from malnutrition, chronic diseases and smoking or any cause of oxidative stress were taken as healthy controls. Blood samples were collected from β-thalassemic patients before and after twelve months of antioxidant vitamins treatment. The patients were treated with higher doses of vitamin E (400–600 mg/day), vitamin C (100 mg/day) and vitamin A (25000 IU/ week). The patients will have history of anemia, abnormal complete blood counts and abnormal whole blood hemoglobin concentration.

The thalassemic patients received transfusion at every three to four weeks interval. Transfusion characteristic and duration of transfusion were similar in all patients. Also thalassemic patients received oral dose of ferriporon (L1) as iron chelator in regular dose (75 mg/kg/day when serum ferritin was less than 2500 μg/L and 100 mg/kg/day when serum ferritin was more than 2500 μg/L). So the patients were under regular blood transfusion and regular chelation therapy at the time of study.

The exclusion criteria were acute or chronic infection, other hematologic disease comorbidity, chronic renal failure, antioxidant or herbal medicine taking, and patients who will be suspected to acquire allergy to vitamins E, C and A. Blood will be drawn from the patients each time just before they received transfusion when hemoglobin values are at their lowest to assess the condition of the patients before they receive exogenous blood. Vitamins E, C and A therapy in β-thalassemic patients started immediately after the first transfusion (after selection of the patients). 3–5 mL of venous blood samples were collected from thalassaemic patients and normal children into heparinized tubes.

DETERMINATION OF ERYTHROCYTES HEMOLYSIS

Percentages of hemolysis were determined by the measurement of hemoglobin (Hb) released from the cells, relative to the total cellular Hb content. The blood was centrifuged at 3000 rpm for 5 minutes and the plasma was removed. The packed erythrocytes were washed three times with 4-fold phosphate-buffered saline (PBS) to remove the plasma remnant. After each procedure, erythrocyte PBS mixture was centrifuged at 3000 rpm for 5 min. 40 μL packed erythrocytes were hemolyzed by adding 5 mL of phosphate buffer (Na_2HPO_4 27.50 mmol/L and KH_2PO_4 13.16 mmol/L, pH 7.3) containing 0.4% Triton-X100. 40 μL packed erythrocytes were added to 5 mL PBS. The percentage of hemolysis was calculated with the following equation:

$$H\% = \frac{A_t}{A_\infty} \times 100 \tag{1}$$

where: A_t , absorbance of the Hb in the supernatant prepared by centrifugation of 40 μL erythrocytes + 5 mL PBS mixture at 3000 rpm, for 10 minutes; A_∞, absorbance of the Hb solution after total hemolysis.

The absorbance of these solutions was measured at 522 nm (the isobestic point for HbO_2 and MetHb). All incubations were done in the air at room temperature.

MULTI-COMPONENT SPECTROPHOTOMETRIC METHOD FOR THE SIMULTANEOUS DETERMINATION OF FOUR HEMOGLOBIN DERIVATIVE CONCENTRATIONS

Concentrations of SHb, MetHb, HbCO and HbO_2 were measured by the multi-component method developed in our laboratory [5] with some modifications. The hemolysate was prepared as described in this method. For absorbance measurements, about 30 μl of the purified hemolysate from normal children is added to 5 mL of temperature equilibrated (25 ºC) phosphate buffer (Na_2HPO_4

27.50 mmol/L and KH_2PO_4 13.16 mmol/L, pH 7.28) containing 0.4% Triton-X100. Whereas 45–65 μL of the purified hemolysate from anemic children were added to the same solution, the concentration of Hb at this extreme dilution is about 3.8×10^{-5} M. The samples were measured and the concentrations calculated as described previously [5] with some modifications regarding the total Hb concentration.

$$C_{\text{total Hb}} = DF \times 1.6114 \times C^{*}_{\text{Hb}} \text{ g dL}^{-1} \tag{2}$$

where DF is the total dilution factor and 1.6114 is the conversion factor for mmol L^{-1} to g dL^{-1}. The total dilution factor can be calculated from the following equation:

$$DF = 1.5 \times \frac{V + 5000}{V} \tag{3}$$

where V is the volume of the added purified hemolysate. The total blood Hb concentration as determined by the multi-component spectrophotometric method by using the last equation was compared with that determined by MetHb cyanide (MetHbCN) method [30].

ALKALINE DENATURATION RATE MEASUREMENT

The blood was centrifuged at 3000 rpm for 5 minutes and the plasma was removed. The packed erythrocytes were washed three times with 4-fold phosphate-buffered saline (PBS) to remove the plasma remnant. After each procedure, erythrocyte PBS mixture was centrifuged at 3000 rpm for 5 min. The packed erythrocytes were brought to 1.5-times the original blood volume with ice-cold 0.4% Triton-X100 to obtain hemolysate. After mixing thoroughly, the hemolysate was centrifuged at 10,000 rpm for 20 minutes to remove erythrocytes ghosts. Dilute a quantity of this Hb solution to a concentration 64 μM with 5 mL distilled water. After adding 25 μL of 10% NH_4OH, read the absorbance at 576 nm (A_0). Add the same volume of Hb solution you added to 5 mL distilled water to 5 mL temperature equilibrated (25 ºC) 0.02 N NaOH to obtain a concentration of 64 μM of Hb. After adding 25 μL of 10% NH_4OH read the absorbance at 576 nm at different times of the alkaline denaturation reaction (A_t). Then the solution is placed in a water path at 37 ºC for 1.5 h. After cooling to room temperature the absorbance is measured at 576 nm (A_∞).

The percentage of undenatured Hb at the time was calculated according to the equation:

$$Hb_t = \frac{A_t - A_\infty}{A_0 - A_\infty} \times 100 \tag{4}$$

where: A_0, absorbance at 576 nm at the start of the reaction; A_∞, absorbance at the end of the reaction; A_t, absorbance at the time t during the reaction.

Since the denaturation of fetal hemoglobin behaves like a monomolecular reaction, the natural logarithm of the percentages of undenatured hemoglobin at different moments plotted against time (in the time interval 10–18 minutes) will form a straight line, the slope of which gives the rate constant of the alkaline denaturation reaction. By extrapolation to 0 times the percentage of fetal Hb in the original blood can be calculated.

DATA ANALYSIS

Data are presented as means ± S.D. Student′s t-test was used for determination of the level of significance of the difference between different groups. The difference is considered significant at $P < 0.05$.

RESULTS

Erythrocytes lose their viability by membrane damage and release hemoglobin into the medium, i.e., hemolysis, which can also be considered as a special case of "interphase cell death". Figure 1 illustrates percentage hemolysis as a function of incubation time. The extent of hemolysis increases proportionally to the incubation time, up to the plateau, starting after the third day up to the sixth day of incubation, after which the extent of hemolysis increases again proportionally to the incubation time. The extent of hemolysis of erythrocytes of β-thalassemic children before treatment with antioxidant vitamins is markedly higher than that of controls at all incubation times, whereas, that of β-thalassemic children after treatment is of intermediate values at all incubation times. As shown in Figure 1**,** the extent of hemolysis of erythrocytes of β-thalassemic children decreases markedly after treatment with antioxidant vitamins, when compared to that before treatment. The results of the mean percentage of hemolysis after 10 days of incubation time for controls and β-thalassemic children before and after treatment with antioxidant vitamins are shown in Figure 2. The mean value of hemolysis for β-thalassemic children before treatment is significantly higher ($P < 0.0001$) than that of controls, whereas, that of β-thalassemic children after treatment is significantly lower ($P < 0.05$) than that of β-thalassemic children before treatment.

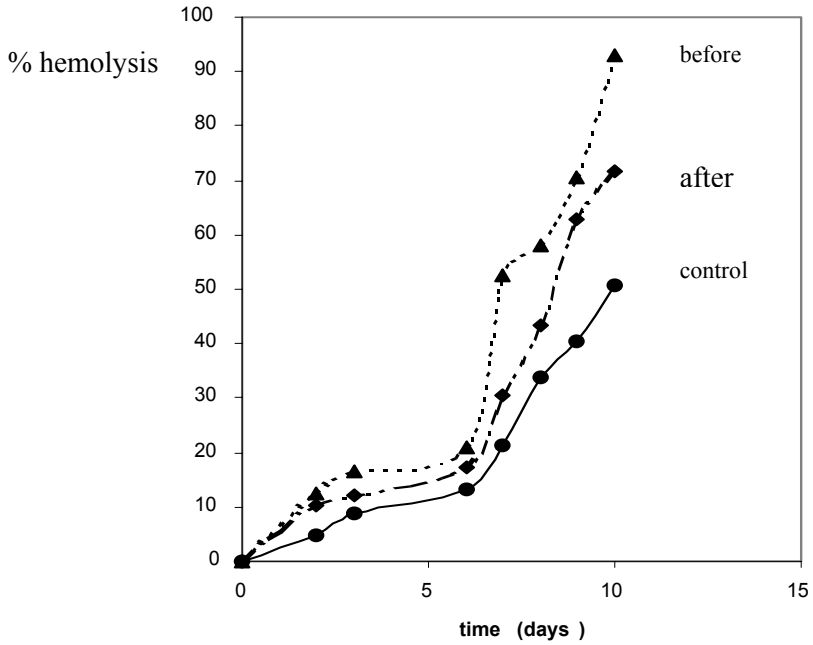

Fig. 1. Percentage hemolysis of human erythrocytes as a function of incubation time for normal and β-thalassemic children before and after treatment.

The mean percentage values of Hbs with different ligands (SHb, MetHb, HbCO and HbO_2) in normal and β-thalassemic blood before and after treatment with antioxidant vitamins are shown in Table 1. Significant increases in the percentages of MetHb and HbCO concomitant with significant decreases in HbO_2 % and HbO_2 concentration are observed in β-thalassemic blood before treatment, when compared to normal blood. Significant decreases in MetHb % and HbCO % concomitant with significant increases in HbO_2 % are observed in β-thalassemic blood after treatment with antioxidant vitamins, when compared before treatment.

Total hemoglobin concentration and the comparative results are shown in Table 2. The values of HbO_2 concentration determined by multiplying the fraction of HbO_2 by the total concentration, as determined by multi-component method, are also shown in this table. The multi-component spectrophotometric method has yielded values of the total blood Hb concentration which is in complete agreement with those determined by using MetHbCN method and showed non significant differences between values of the two methods. A highly significant increase in the level of HbO_2 of β-thalassemic patients after treatment with antioxidant vitamins, as compared with that before treatment, was observed.

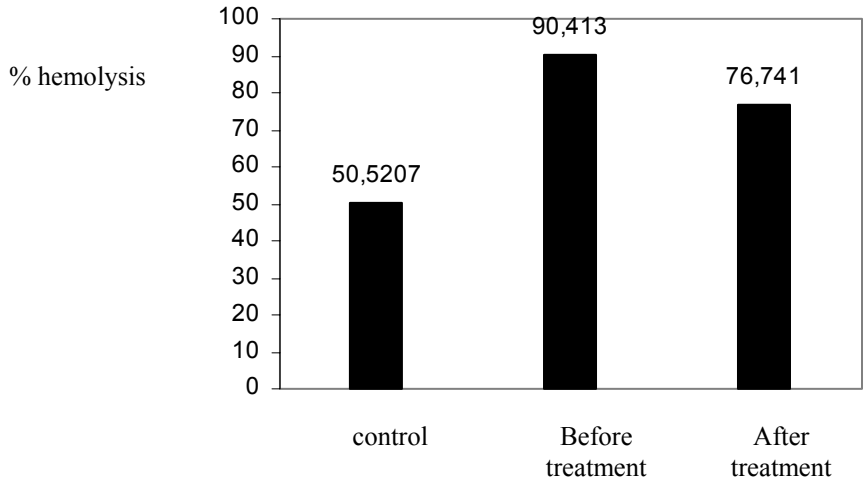

Fig. 2. Percentage of erythrocytes hemolysis after 10 days incubation for normal and β thalassemic children before and after treatment. * *P* vs. normal, ** *P* vs. before treatment values.* $P < 0.0001$, ** $P < 0.05$.

Table 1

Percentages of inactive hemoglobins and the active Hb (in the HbO_2 form) in the human blood from normal and β-thalassemic children before and after treatment

Group	**SHb (%)**	**MetHb (%)**	**HbCO (%)**	**HbO_2 (%)**
Control (n = 8)	0.257±0.097	0.729±0.228	1.065±0.447	97.948±0.525
Before treatment	(NS^1)	*	**	*
(n = 10)	0.199±0.191	2.896±0.827	2.220±0.832	94.684±0.891
After treatment	(NS^2)	***	***	****
(n = 10)	0.161±0.169	2.078±0.249	1.254±0.676	96.505±0.757

Values are means ± SD. n is the number of samples. *, ***P* vs. normal, ***, ****, *****P* vs. before treatment values. NS^1 (not significant) vs. normal, NS^2 (not significant) vs. before treatment values. * $P < 0.00005$, ** $P < 0.005$, ***$P < 0.01$, ****$P < 0.0001$, ***** $P < 0.001$.

Table 2

Total Hb concentration by the multi-component spectrophotometric method, in comparison to the MetHbCN method

Group	**Total Hb concentration by the multi-component method (g/dL)**	**Total Hb concentration by the MetHbCN method (g/dL)**	**HbO_2-concentration (g/dL)**
Control (n = 8)	14.88±1.02	14.87±1.09	14.6±0.97
Before treatment (n = 10)	7.5±0.88	7.62±0.77	6.516±0.373
After treatment (n = 10)	8.36±0.94*	8.53±0.83*	7.884±0.897**

Values are means ± SD. *, ** P vs. before treatment, * $P < 0.025$, ** $P < 0.0005$

First-order rate plots for alkaline denaturation of control and β-thalassemic hemoglobins, before and after treatment with antioxidant vitamins, are shown in Figure 3, respectively. As shown in these figures, two straight lines with different slopes are observed. The straight line with a higher slope represents the alkaline-denaturation reaction of the adult Hb (HbA), while those with slower slopes represent the alkaline-denaturation reaction of the fetal Hb (HbF). The slopes of these sraight lines determine the rate constants for the alkaline-denaturation reaction.

The results of the alkaline-denaturation rates of HbA and HbF, for control and β-thalassemic children before and after treatment with antioxidant vitamins, are shown in Table 3. These results reveal insignificant increases in the rate of alkaline denaturation of HbA and HbF from β-thalassemic children before treatment, when compared to controls and insignificant increases in β-thalassemic children after treatment with antioxidants, when compared to those before treatment. The rates of alkaline denaturation of adult Hb are 4.213, 4.318 and 5.112 times those of fetal Hb, for controls, β-thalassemic children before and after treatment, respectively. By extrapolation of the straight lines (in the time interval 10–18 minutes) to 0 times the percentage of fetal hemoglobin in original blood can be calculated.

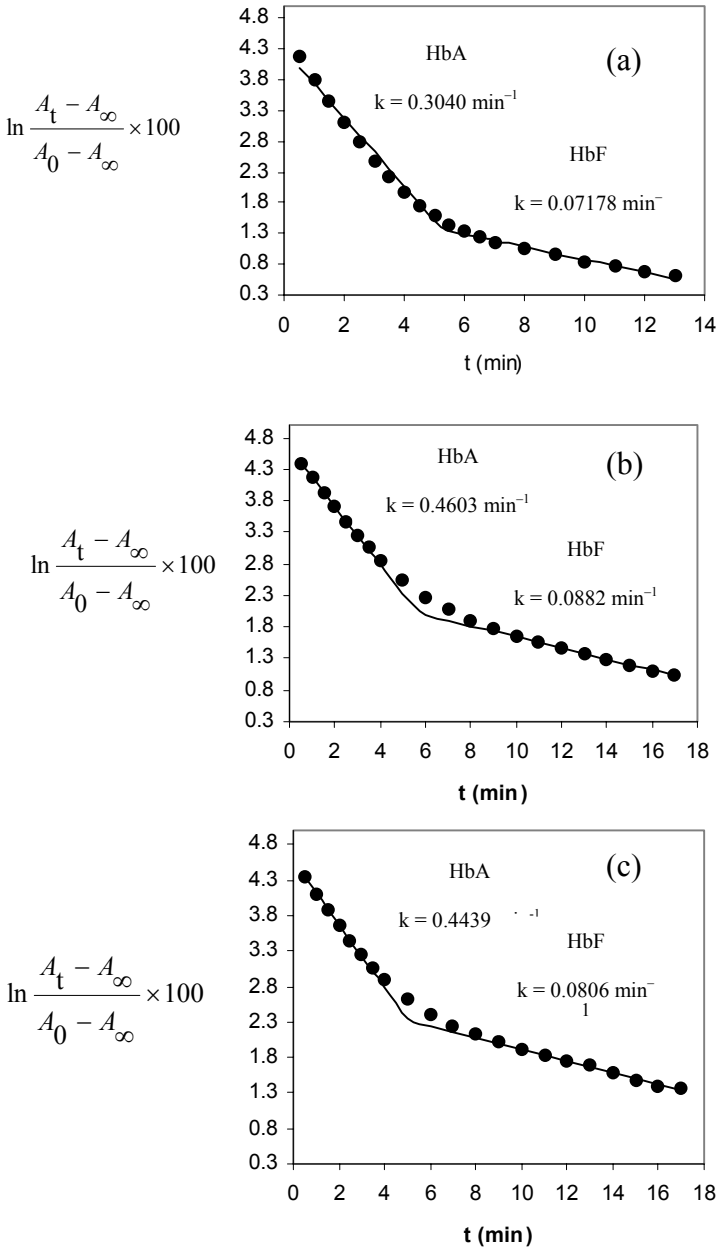

Fig. 3. Kinetics of alkaline denaturation of Hb from (a) normal, β-thalassemic children (b) before, and (c) after treatment with antioxidant vitamins.

Table 3

Rates of alkaline denaturation of hemoglobins extracted from normal and β-thalassemic children before and after treatment

Group	k **(min^{-1})**	
	HbA	**HbF**
Control (***n*** **= 3)**	0.300 ± 0.0374	0.0712 ± 0.0066
Before treatment (***n*** **= 3)**	0.3299 ± 0.0889 (NS^1)	0.0764 ± 0.0102 (NS^1)
After treatment (***n*** **= 3)**	0.3962 ± 0.0818 (NS^2)	0.0775 ± 0.0052 (NS^2)

Values are means ± SD. NS^1 (not significant) vs. normal, NS^2 (not significant) vs. before treatment values.

DISCUSSION AND CONCLUSIONS

Free radicals attack various biological molecules, membranes and tissues to induce free radical-mediated chain oxidations. It has been found that aqueous peroxyl radicals induce the oxidation of phospholipid liposomal membranes, erythrocyte membranes. For example, the aqueous peroxyl radicals attack erythrocyte membranes and induce the oxidation of lipids and proteins to cause possible hemolysis [35]. The extent of hemolysis was proportional to the amount of free radical attack [23]. Ascorbic acid suppressed the hemolysis dose dependently, that is, as the amount of ascorbic acid increased, the hemolysis took place later.

Ascorbic acid is not only a chain-breaking antioxidant; tocopherol (vitamin E), ubiquinol, β-carotene, uric acid, thiols, albumin, and bilirubin also act as chain-breaking antioxidants [15]. In particular, uric acid is known to act as a potent, hydrophilic antioxidant [3]. Wayner *et al.* [34] measured the total radical-trapping capacity of antioxidants in human plasma and found that their contributions were 35–65% from urate, 0–24% from ascorbate, 5–10% from vitamin E, and 10–50% from plasma proteins. Stocker *et al* [28] observed that antioxidants reacted with peroxyl radical in the order of ascorbate, albumin-bound bilirubin, and urate.

Vitamin E, particularly α-tocopherol, functions *in vivo* as a lipid soluble, chain breaking antioxidant [7, 17, 18] and is a potent peroxyl radical scavenger [7]. When lipid hydroperoxides are oxidized to peroxyl radicals ($ROO^{\cdot}$), these react 1000-times faster with α-tocopherol (TOH) than with PUFA (RH). The phenolic hydroxyl group of the chromanol ring reacts with an organic peroxyl radical to form the corresponding organic hydroperoxide and the vitamin E radical ($TO^{\cdot}$) [8]. In the presence of vitamin E:

$$ROO^{\cdot} + TOH \longrightarrow ROOH + TO^{\cdot} \quad (5)$$

In the absence of vitamin E:

$$ROO^{\cdot} + RH \longrightarrow ROOH + R^{\cdot} \quad (6)$$

$$R^{\cdot} + O_2 \longrightarrow ROO^{\cdot} \quad (7)$$

In this way, α-tocopherol, for example, acts as a chain-breaking antioxidant, preventing further auto-oxidation of PUFA. The tocopheroxyl radical (TO.) reacts with vitamin C (or other reductants serving as hydrogen donors, AH), thereby oxidizing the latter and returning vitamin E to its reduced state [9].

$$TO^{\cdot} + AH \longrightarrow TOH + A^{\cdot} \quad (8)$$

Biologically important hydrogen donors, which have been demonstrated *in vitro* to regenerate tocopherol from the tocopheroxyl radical, include ascorbate (vitamin C) and glutathione [21, 23].

This study showed that the extent of hemolysis of erythrocytes of β-thalassemic children before treatment with antioxidant vitamins is markedly higher than that of controls at all incubation times, whereas, that of β-thalassemic children after treatment is of intermediate values at all incubation times. The extent of hemolysis of erythrocytes of β-thalassemic children decreases markedly after treatment with antioxidant vitamins, when compared to that before treatment. Desouky *et al.* [13] demonstrated that the high hemolytic rate can be easily detected by simple hemolysis test, where the rate of hemolysis for thalassemic RBCs exceeds the normal rate. In their study the rate of hemolysis of β-thalassemia was approximately double the rate of normal RBCs. Thasinas *et al.* [29] reported that, theoretically, vitamin E and glutathione are the red cell protective antioxidants; the increasing level of both of them should decrease intravascular hemolysis.

The decrease in the concentration of vitamins E and C is accompanied with a high level of lipid peroxidation product (MDA) (unpublished results) and consequently can account for the high percentage of hemolysis of erythrocytes in β-thalassemic patients before treatment observed in the present study. The increase in the concentrations of vitamins E and C after treatment of β-thalassemic patients with these antioxidants can account for the decrease in the level of lipid peroxidation product and membrane damage and consequently the decrease in the percentage of hemolysis of erythrocytes observed in the present study.

When red cells reach the end of their life due to aging or defects, they are broken down, the hemoglobin molecule is broken up and the iron gets recycled. When the porphyrin ring is broken up, the fragments are normally secreted in the bile by the liver. This process also produces one molecule of carbon monoxide (CO) for every molecule of heme degraded [16]; this is one of the few natural

sources of carbon monoxide production in the human body, and is responsible for the normal blood levels of carbon monoxide and carboxyhemoglobin (HbCO) even in people breathing pure air. This may explain the higher HbCO level that accompanies the higher rate of hemolysis and Hb and heme degradation in β-thalassemic children and the decrease in its level that accompanies the decrease in hemolysis after treatment with antioxidants.

The binding of oxygen is affected by molecules such as carbon monoxide (CO). CO competes with oxygen at the heme binding site. Hemoglobin binding affinity for CO is 200 times greater than its affinity for oxygen, meaning that small amounts of CO dramatically reduce hemoglobin's ability to transport oxygen. Hemoglobin bonds to carbon monoxide preferentially (200:1 more so) compared to bonding to oxygen, so effectively HbCO will not release the carbon monoxide, and therefore hemoglobin will not be available to transport oxygen from the lungs to the rest of the body.

Since the inactive components of Hb (SHb, MetHb and HbCO) are unable to transport oxygen, the net concentration of active functional Hb (in the HbO_2 form) is an indicator of the actual degree of anemia. The increase in the percentages of MetHb and HbCO components together with an increase in the rate of hemolysis may explain the decrease in HbO_2 concentration in β-thalassemic children before treatment, when compared to controls. The decrease in MetHb% and HbCO% together with a reduction in the rate of hemolysis of erythrocytes may explain the increase in the concentration of HbO_2 observed after treatment of β-thalassemic patients with antioxidants. This agrees with Ruchaneekorn *et al.* [27] who stated that the percentage of MetHb was significantly decreased ($P < 0.05$) after administration of curcuminoids for 12 months, while there were no changes in Hb levels, but in our study there was improvement of hemoglobin concentrations in thalassemic patients.

Methemoglobin is a form of oxygen-carrying protein hemoglobin, in which the iron in the heme group is in the Fe^{3+} state, not the Fe^{2+} of normal hemoglobin. Hemoglobin can be considered to exist in active and inactive states. When the iron atom is in the ferrous form, the protein is active and can bind oxygen reversibly. The oxidation to the ferric form (MetHb) leads to an inactive protein. Methemoglobin is unable to carry oxygen. Healthy people may not have many symptoms with methemolgobin levels <15%, however patients with co-morbidities such as anemia, cardiovascular disease or presence of other abnormal hemoglobin species (e.g. carboxyhemoglobin and sulfhemoglobin) as in β-thalassemic children may experience moderate to severe symptoms at much lower levels (as low as 3–5%).

The increase in oxidative stress in β-thalassemic patients can account for the increase in the level of MetHb produced through HbO_2-autoxidation reactions [33]. The decrease in oxidative stress as a result of antioxidants treatment of β-thalassemic patients can account for the decrease in the level of MetHb and the increase in the level of HbO_2 observed in the present study. The increased HbO_2

concentration and improved anemia may be caused not only by vitamins E and C treatment but also by vitamin A supplementation, since according to previous studies, vitamin A supplementation increases erythropoietin and hemoglobin concentrations in children with poor vitamin A status [24, 36].

Since Vechio's [32] discovery that fetal hemoglobin is found in large amounts in the blood of patients with Cooley's anemia, the interest of hematologists in this hemoglobin has increased. It is now known that also in patients with sickle-cell syndrome anemia (and perhaps in other hereditary hemolytic anemias) fetal hemoglobin may occur [1]. An accurate method for the determination of small percentages of fetal hemoglobin is therefore important. The usual technique for the determination of abnormal hemoglobins is paper electrophoresis. Quantitatively the results are not very accurate. With this technique, moreover, the separation of adult and fetal hemoglobin is poor. Far better are the results obtained with the alkali denaturation method. The great differences in resistance to alkali between fetal and adult hemoglobin makes it possible to estimate the amount of fetal hemoglobin present in the mixture of the two. The results of this method, suggested here in this study for determination of the percentage of fetal hemoglobin, revealed values in the range (12.5–45.38%) and (9.289–33.99%) for β-thalassemic children before and after treatment with antioxidants, respectively.

In conclusion, the treatment of β-thalassemic major patients with the vitamin A, vitamin C, and vitamin E together improves the antioxidant status of these patients and protects erythrocytes from the damage resulting from oxidative stress and hence reducing the rate of hemolysis and improves the total Hb concentration. Treatment of β-thalassemic major patients with antioxidant vitamins has no effects on the rate of alkaline denaturation of Hb, whereas it reduces the levels of HbCO and MetHb therefore increasing the HbO_2 concentration which represents the actual measure of the degree of anemia.

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