EFFECTS OF ANTIOXIDANT VITAMINS ON THE OXIDANT/ANTIOXIDANT STATUS AND LIVER FUNCTION IN HOMOZYGOUS BETA-THALASSEMIA

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Abstract. The present work is concerned with the study of the effects of antioxidant vitamins on antioxidant status and liver function in homozygous β-thalassemic patients. The patients were treated with vitamins E, C and A for twelve months. With respect to antioxidant vitamins, before treatment there were deficiencies in these vitamins in thalassemic patients as compared with healthy controls. Also, before treatment there was a significant elevation in the malondialdehyde (MDA) concentration, and a significant deficiency in levels of reduced glutathione (GSH). After treatment, patients with β-thalassemia major exhibited significant improvements in the levels of non-enzymatic parameters as compared with the levels of these parameters before treatment. Also, MDA significantly decreased and reduced glutathione was highly increased after treatment. Also, values of Hb and ferritin showed improvements of their values after treatment. The results of enzymes showed that thalassemic major children suffer from high levels of ALT, AST, glutathione peroxidase, and superoxide dismutase enzymes activities before vitamins treatment. The activities of ALT, AST, GPx, and SOD decreased significantly, also the activities of catalase and glutathione reductase significantly increased in β-thalassemic patients after treatment compared with their activities before treatment. Finally, it seems clear that treatment of β-thalassemic patients with antioxidant vitamins serves to improve the healthy status of the patients through an enhancement of the levels of antioxidants and reduction of the oxidative damage, and hence improve the hemoglobin levels and liver function.

Key words: β-thalassemia, vitamin E, vitamin C, vitamin A, GSH, MDA, ferritin, catalase, SOD, glutathione peroxidase, glutathione reductase, ALT, AST, 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

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thalassemia syndromes. In Egypt, β-thalassemia is the commonest cause of chronic 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 [16, 32].

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) [5].

In β-thalassemia syndromes, decreased or impaired biosynthesis of beta-globin 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 [29]. 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 [27].

Vitamins A, E & C, carotene and glutathione (GSH) provide antioxidant defenses 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 [3]. Due to increased consumption low plasma levels of tocopherol, a chain breaking antioxidant may induce lipid peroxidation within the red blood cells and consequently hemolysis [18]. The degree of lipid peroxidation in the organism can be evaluated by malonyldialdehyde (MDA), which is the breakdown product of lipid peroxidation [12]. 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 [14].

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 [15]. 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 [15]. In this study no improvements in vitamins E and C concentrations were 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 [23]. However, the interaction between the two vitamins and their effects on the oxidative stress and antioxidant status in β-thalassemia has not been studied yet. Moreover, the effects of vitamin A treatment on the liver function and hemoglobin levels in β-thalassemics have not been evaluated. A recent study reported lower levels of vitamin A in homozygous β-thalassemias than in controls [42].

Therefore, we aimed to study the effects of antioxidant vitamins treatment on the oxidative stress, antioxidant status, Hb concentration and liver function in patients with homozygous β-thalassemia.

MATERIALS AND METHODS

PATIENTS AND BLOOD COLLECTION

The current study involved thirty homozygous β-thalassemic patients aged 4–17 years (15 males and 15 females) admitted to Hematology and Oncology Department, Pediatric Hospital, Ain Shams University. A group of 20 children in the same age and sex and free from malnutrition, chronic diseases and smoking or any cause of oxidative stress were taken as healthy controls.

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 vitamins E, C and A allergy. Blood

will be drawn from the patients each time just before they received transfusion when hemoglobin values are at their lowest. This was 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). A total of 30-thalassemic children patients were treated with oral dose of vitamin E (400–600 mg/day) (400 mg/day in patient weighed less than 20 kg and 600 mg/day in patient weighed at least 20 kg), low dose of vitamin C (100 mg/day) and vitamin A (25000 IU/week) for twelve months. Blood samples were collected from all patients before and after 6 months from the treatment and after twelve months from the treatment.

3–5 mL of venous blood samples were collected from thalassaemic patients and normal children in the same age and sex, withdrawn into one EDTA and one heparin tubes and collected under complete aseptic conditions from all children under investigation. The EDTA whole blood sample tube was used for hemoglobin determination, complete blood picture, hematocrit, and glutathione reduced (GSH). The heparinized whole blood tube was centrifuged for 10 minutes at 3000 rpm and the plasma was separated and used in ALT, AST, malondialdehyde (MDA), ascorbic acid (Vitamin C), and catalase enzyme determination. The separated erythrocytes were washed four times with 3 mL 0.9% saline solution and centrifuged for 10 minutes at 3000 rpm after each wash, then hemolyzed by adding approximately 1.5 volume of cold water for superoxide dismutase (SOD), glutathione reductase, and glutathione peroxidase determination. All kits were purchased from the Biodiagnostic Company, Cairo, Egypt.

DETERMINATION OF BIOCHEMICAL PARAMETERS

Hemoglobin was colorimetrically determined according to the method of Van Kampen and Zijlstra [41]. Alanine transaminase (ALT) and aspartate transaminase (AST) activities were determined spectrophotometrically by the method of Reitman and Frankel [35]. Reduced glutathione in the blood was determined spectrophotometrically according to the method of Beutler *et al.* [7]. Malondialdehyde (MDA) plasma level was determined spectrophotometrically by the method of Satoh [37]. Superoxide dismutase activity in cell lysate was determined spectrophotometrically according to the method of Nishikimi *et al.* [31]. The activity of erythrocyte glutathione reductase (GSH-R) was determined spectrophotometrically by the method of Goldberg and Spooner [19]. Glutathione peroxidase activity in cell lysate was determined spectrophotometrically by the method of Paglia and Valentine [33]. Catalase activity in plasma was determined spectrophotometrically by the method of Aebi [1]. The plasma level of ascorbic acid (vitamin C) was determined spectrophotometrically according to the method of Harris and Ray [22].

DETERMINATION OF α-TOCOPHEROL (VITAMIN E) AND RETINOL (VITAMIN A) BY HPLC

High-performance liquid chromatography (HPLC) has proven to be a very useful technique for measuring of α-tochopherol and retinol. The advantages of HPLC analysis include the small sample size requirement, the nondestructive nature and speed of the assay, and the highly accurate and reproducible separation of the different compounds [25].

HPLC reference standard, including α-tocopherol and retinol, were purchased from Sigma Chemical Company (Poole, Dorset, UK). HPLC-grade methanol, chloroform, and acetonitrile (JT Baker Chemical Company, Phillipsburg, NJ) were filtered through a Millipore FH 0.5-μm filter (Millipore Corp., Bedford, Mass.) and degassed under vacuum daily prior to use.

Retinol and α-tochopherol standards were prepared in 100% ethanol. Concentrations of the standard solutions were determined using the following extinction coeffecients (E 1%/cm): retinol, 1780 at 325 nm and α-tocopherol, 75.8 at 292 nm.

200 μL of 100% ethanol was added to 200 μL of plasma. The samples were vortexed for 15 seconds, 400 μL of hexane was added, and mixture was vortexed for 60 seconds. The samples were centrifuged for 5 minutes at 1000 rpm in a bench-top centrifuge to aid in the solvent separation. Following centrifugation, 300 μL of the hexane layer were removed and evaporated to dryness under a stream of N_2, and the residue was redissolved in 100 μL of ethanol. Finally, 40 μL samples were injected onto the column. All handling of samples and standards was done under dimmed light and samples were kept on ice whenever possible during the procedure. Ethanol and hexane, like the HPLC solvents, were filtered and degassed prior to use. The multiple-solvent system used in the reverse phase HPLC method was 100% methanol for 8 minutes at 1mL/min and then the system was automatically switched to a methanol: acetonitrile: chloroform (47:42:11) solvent mix with a flow rate of 2 mL/minute.

STATISTICAL ANALYSIS

Descriptive data were analyzed by percentage, mean, and standard deviation. Groups comparisons involved analysis of variance (ANOVA) followed by appropriate tests significance. The differences in the continuous variables were compared by using the paired t-test (SPSS for Window version 11.0, Chicago, USA). A *P*-value of < 0.05 was considered statistically significant.

RESULTS

As shown in Table 1, before treatment with combined three vitamins (A,C, and E), there was deficiency in these vitamins in thalassemic patients compared with healthy controls and their values were 3.1, 4, and 2.6 times respectively lower than in healthy controls. Also, malondialdehyde concentration was 3.4 times higher in β-thalassemia patients than in healthy controls. While a highly significant deficiency

Table 1

Values of vitamin A, vitamin C, vitamin E, malondialdehyde, reduced glutathione, hemoglobin, and ferritin of β-thalassemic children before and after vitamins treatment and healthy controls

Parameter	**Healthy controls (*n* = 20)** (Mean±SD)	**β-thalassemic patients (*n* = 30)**		
		Before treatment (Mean±SD)	**After 6 months treatment** (Mean±SD)	**After 12 months treatment** (Mean±SD)
Vitamin A (µmol/L)	1.43±0.55	0.45±0.25 *P*<0.05	0.87±0.38 *P*<0.001	1.4±0.6 *P*<0.001
Vitamin C (mg/L)	15.5±6.2	3.84±1.7 *P*<0.001	6.45±2.2 *P*<0.001	8.7±2.5 *P*<0.002
Vitamin E (µmol/L)	14.97±4.55	5.7±2.2 *P*<0.001	8.2±3 *P*<0.001	11.6±4.1 *P*<0.001
Malondialdehyde (nmol/L)	2.3±1.2	7.87±1.82 *P*<0.001	5.75±1.65 *P*<0.001	4.24±1.3 *P*<0.0025
Reduced glutathione (mg/dL)	28.27±4.96	9.82±3.3 *P*<0.001	7.06±5.9 *P*<0.001	27.186±6.39 *P*<0.0001
Hemoglobin (g/dL)	13.5±1.4	6.8±1.15 *P*<0.0001	7.25±1.17 *P*<0.001	7.8±1.1 *P*<0.001
Ferritin (µg/L)	–	2802.45±1364.3	2857.55±1054.6 *P*<0.01	2499.2±939.1 *P*<0.05

in the level of reduced glutathione which is 3.2 times lower than in healthy controls is noticed. After six months of combined three vitamins treatment patients with β-thalassemia major exhibited a significant improvement in the levels of non-enzymatic parameters in comparison with the levels of these parameters before treatment. After this period, the levels of these parameters could not be normalized in most thalassemic patients. After a period of twelve months of vitamins treatment, improvements in all non-enzymatic antioxidants levels are noticed when compared with untreated β-thalassemia patients. MDA was decreased significantly after treatment, as compared to before treatment. Reduced glutathione increased significantly after treatment as compared to before treatment. Also, values of Hb and ferritin showed improvements of their values after treatment.

Table 2

Activities of ALT, AST, catalase, glutathione peroxidase, glutathione reductase, and superoxide dismutase of β-thalassemic children before and after vitamins treatment and in healthy controls

Enzyme	Healthy controls (n = 20) (Mean±SD)	B-thalassemic patients (n = 30)		
		Before treatment (Mean±SD)	After 6 months treatment (Mean±SD)	After 12 months treatment (Mean±SD)
ALT (IU/L)	19.85±9.42	60.46±22.42 P<0.001	49.1±15.37 P<0.001	38.13±13.45 P<0.003
AST (IU/L)	18.25±9.44	64.1±19.52 P<0.001	54.7±19.2 P<0.001	44.1±16.4 P<0.001
Catalase (U/L)	118.27±23.17	42.86±17 P<0.001	59.26±17.23 P<0.001	76.14±16.22 P<0.001
Glutathione peroxidase (U/L)	15.89±5.6	43.89±9.5 P<0.001	38.05±14.22 P<0.001	23.5±8.24 P<0.05
Glutathione reductase (U/L)	12.43±3.85	3.36±1.7 P<0.001	5.61±1.9 P<0.001	8.22±2.33 P<0.001
Superoxide dismutase (U/mL)	102.1±40.5	308±66.2 P<0.001	242.2±51 P<0.001	177±51.4 P<0.001

As shown in Table 2, thalassemic major children suffer from high levels of ALT, AST, glutathione peroxidase, and superoxide dismutase enzymes activities before vitamins treatment. The values of these enzymes are 3, 3.5, 2.8, and 3 times respectively higher than in healthy controls. Also, there were low activities of catalase and glutathione reductase. The decrements of these enzymes activities are 2.75 and 3 times respectively lower than in the healthy controls.

After six months of vitamins treatment, the activities of these enzymes were found to be improved in comparison with their activities before treatment but these activities did not reach normal activities in most patients. The activities of ALT, AST, GPx, and SOD decreased significantly, but they did not reach the normal range compared with healthy controls. Also the activities of catalase and glutathione reductase were found to be significantly increased in β-thalassemic patients as compared with their activities before treatment. After twelve months of vitamins treatment, there are highly significant improvements of enzymatic antioxidants parameters as compared with before treatment enzyme activities. ALT, AST, and SOD highly significantly decreased, while the decrease of glutathione peroxidase activities is significant. On the other hand, the increase of catalase and glutathione reductase activities after treatment is highly significant (Table 2).

DISCUSSION AND CONCLUSIONS

In the present study, thirty β-thalassemic patients received oral dose of vitamins treatment, vitamin E (400–600 mg/day), vitamin C (100 mg/day) and vitamin A (25000 IU/ week) for 12 months. Blood samples were collected from all patients before and after six months from the treatment and after twelve months from the treatment. The blood samples were tested for biochemical parameters.

Before treatment with combined three vitamins (A,C, and E), there was deficiency in these vitamins in thalassemic patients as compared with healthy controls and their values were 3.1, 4, and 2.6 times respectively lower than in healthy controls. In agreement with these results, Veena *et al.* [42] reported low levels of plasma vitamin E in β-thalassemic patients. On the basis of their observations, Stocks *et al.* [38] concluded that vitamin E was, by no means, the only factor for protection against anti-oxidant stresses. However, vitamin C has a particular role in vitamin E recycling and some reports have found vitamin C deficiency in thalassemia patient [34].

Also in this study, there was high elevation in the malondialdehyde concentrations, which is 3.4 times higher in β-thalassemia patients than in healthy controls. These results agree with previous studies, which reported increased plasma malonyldialdehyde (MDA) level, as measured by the thiobarbituric acid reaction substance (TBARS) methods, in β-thalassemia patients [18, 30, 39]. MDA is a good indicator of oxidative damage. In addition, malondialdehyde (MDA), a product of lipid peroxidation, is generated in excess amounts in β-thalassemia. MDA is a bifunctional reagent and has been reported to crosslink several cell constituents including membrane components. A cross-linked 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 [26]. In one of the previous studies, free and total MDA was found to be higher in regularly transfused thalassemia major patients than in the thalassemia intermedia patients [13]. As a result of continuous blood transfusions, the patients might be subjected to peroxidative tissue injury by the secondary iron overload. These findings might support the idea of iron overload in β-thalassemia leads to an enhanced generation of reactive oxygen species and oxidative stress.

The present study reported a deficiency in levels of reduced glutathione, which is 3.2 times lower than in healthy controls. These results go hand in hand with a previous study [17], which suggested that glutathione (GSH) is a major interacellular reducing agent, is very sensitive to oxidative pressures and has several important functions such as: protection against oxidative stress, regulation of gene expression, induction of apoptosis, activation, and proliferation in T lymphocytes.

After six months of combined three vitamins treatment, patients with β-thalassemia major exhibited significant improvements in the levels of non-enzymatic parameters in comparison with the levels of these parameters before treatment. After this period, the levels of parameters could not be normalized in most thalassemic patients. These results agree with Thasinas *et al.* [40] who concluded that**,** after the period of treatment, vitamin C and vitamin E increased significantly but could not be normalized. This was due to critically excessive oxidative stress. After supplementation of vitamin C and vitamin E, the authors found that glutathione level was increased. Therefore, vitamin C supplementation in these patients was proved to have benefit. The authors suggested that low dose vitamin C may not be contraindication in β-thalassemia patients who are at risk of vitamin C deficiency. Glutathione is an important antioxidant in red blood cell membrane and necessary in vitamin C recycle pathway [11].

In this study MDA level was reduced after treatment with the combined vitamins. Das *et al.* [15] suggested that erythrocyte membrane from β-thalassemic patients showed high level of lipid peroxidation when compared to 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.

After a period of twelve months of vitamins treatment, there was improvement in all non-enzymatic antioxidants levels as compared with untreated β-thalassemia patients. The vitamin A increased with highly significant change and reached normal values. Vitamin C and vitamin E showed improvement in their levels. Also, MDA decreased significantly, reduced glutathione in the last of treatment period was highly increased, like Thasinas *et al.* [40], who found that after supplementation of vitamin C and vitamin E, glutathione level was increased. Therefore, vitamin C supplementation in these patients was proved to have benefit. Ruchaneekorn *et al.* [36] suggested that the levels of H_2O_2-induced RBC MDA were significantly higher in the β-thalassemic patients than in normal subjects and reduced significantly following vitamins treatment up to 6 months and highly significantly after twelve months of treatment.

In the current study, the levels of the hemoglobin of β-thalassemic patients were evaluated after six and twelve months vitamins treatment and showed a significant increase during treatment compared with before treatment. Das *et al.* [15] revealed that 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 the thalassemic patients the Hb levels, even after vitamin E treatment, never reach the normal values and agree with the present study. Ruchaneekorn *et al.* [36] found no change in Hb levels *in vitro* study, curcuminoids showed the protective effect of RBC from free radical-induced hemolysis in a concentration-dependent manner. However, this effect *in vivo* may be uncertain because of extremely low bioavailability as a consequence of poor solubility of curcumin in an aqueous condition and rapid

metabolism in liver, but in the present study there is improvement in Hb levels in treated thalassemic patients with vitamins as antioxidants.

In the present study thalassemic major children suffer from high levels of ALT, AST, glutathione peroxidase, and superoxide dismutase enzymes activities before vitamins treatment. Also, there was low activity of catalase and glutathione reductase and the decrease of these enzymes activities were 2.75 and 3 times respectively lower than in the healthy controls. An agreement comes from Kassab-Chekir *et al.* [24] who stated that, in the liver, lipid peroxidation is associated with impairment of membrane-dependent functions of mitochondria and lysosomes. In fact, iron overload would impair hepatic mitochondrial respiration primarily through a decrease in cytochrome c oxidase activity, and hepatocellular calcium homeostasis may be compromised through damage to mitochondrial and microsomal calcium sequestration [8]. Haj *et al.* [20] found no significant perturbations in hepatic exploration except an increase of transaminases in β-thalassemia patients in agreement with our study. Also, Das *et al.* [15] reported that, the erythrocyte catalase activity significantly decreased in β-thalassemic patients with the degree of inhibition slightly higher (22.5%) in β-thalassemic patients compared to normal subjects. Glutathione reductase (GR), another important antioxidant enzyme, remained depressed in β-thalassemic patients when compared to normal subjects.

Erythrocytes are protected from oxidative stress by intracellular enzymes such as superoxide dismutase and several other constituents such as vitamin E. SOD is a preventive antioxidant. Increased SOD activities were found in the patients with β-thalassemia as reported previously [9]. Both β-thalassemia and accompanying iron overload lead to *in vivo* lipid peroxidation and the compensatory increase in the antioxidant enzyme levels of SOD and glutathione peroxidase (GPx). This is in agreement with the present study. The significant increased catalytic activities of SOD and GPx in β-thalassemic erythrocytes were found when compared with healthy subjects and β-thalassemic carriers [10]. Increased SOD activity was probably due to an increase in the proportion of younger red blood cells, and the compensatory mechanism after increased oxidant stress [18]. The antioxidant status in β-thalassemia/Hb E patients was investigated by direct measurement of cytoprotective enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) inside RBC. The activities of both enzymes in the patients were significantly increased in response to elevated RBC oxidative stress. The upregulation of SOD activity protects the thalassemic RBC by scavenging superoxide radicals and producing more hydrogen peroxide (H_2O_2), which is removed by GPx [36]. Removal of toxic oxygen metabolites is the putative function of antioxidant enzymes such as SOD and GPx. It has already been demonstrated that oxidative stress induces antioxidative enzymes, including SOD and GPx [28]. The increased activity of SOD in β-thalassemia may be involved in scavenging the superoxide radical (O_2^-), thereby producing more hydrogen

peroxide in the erythrocytes [10]. The increased activity of GPx in β-thalassemia may be involved in detoxifying hydroxyl radical ($OH^{\bullet}$). This finding suggests that high iron produces an oxidative stress in cells, which respond by increasing their antioxidant defenses. The increase of intracellular antioxidant enzymes might be hypothesized to be a direct effect of increased intracellular iron on gene expression. However, such increased enzyme activities (SOD and GPx) were not sufficient to counteract radical oxidative species since there was a significant increase of TBARS in β-thalassemia compared to controls.

After six months of vitamins treatment, the activities of these enzymes were found to be improved in comparison with their activities before treatment but the activities of these enzymes could not reach normal activities in most patients of thalassemia major. The activities of ALT, AST, GPx, and SOD decreased significantly, but not normalized in comparison with healthy controls , also the activities of catalase and glutathione reductase were found to be significantly increased in β-thalassemic patients compared with their activities before treatment.

After twelve months of vitamins treatment, there was highly significant improvement of enzymatic antioxidants parameters as compared with before treatment enzyme activities. ALT, AST, and SOD significantly decreased. On the other hand, the increase of catalase and glutathione reductase activities was highly significant. These results are in complete agreement with those of Ruchaneekorn *et al.* [36] who reported that curcuminoids treatment for 12 months significantly decreased the activities of SOD and GPx in RBC. Also, Das *et al.* [15] reported that a significant improvement in catalase activity was evident after treatment of the patients with vitamin E for four weeks.

Vitamin A plays an essential role in a large number of physiological functions that encompass vision, growth, reproduction, hematopoiesis, and immunity. Vitamin A deficiency has been found to cause oxidative damage to liver mitochondria in rats [6]. Vitamin A has also been found to protect against chemical induced lipid peroxidation in the heart, brain and liver. This study showed improvements of liver enzymes (ALT with $P<0.001$ and AST with $P<0.001$), which reached normal values in most patients due to the presence of vitamin A which had a role in improvement of liver enzymes.

In the current assay, all β-thalassemia major patients had deficiency in all enzymatic and non-enzymatic antioxidants levels before the combined vitamins (A, C, and E) treatment. After six months of treatment, there was an increase in vitamin A levels in 57% of thalassemia major patients, an increase in vitamin C levels in 57% of thalassemic patients, also the increase in vitamin E levels appeared in 33% of thalassemic patients, but this value did not reach a significant level. After twelve months of treatment, there was a significant increase in all thalassemic patients. The results revealed an increase in vitamin A levels in 87% of treated patients, an increase in vitamin C levels in 83% of patients, and an increase in vitamin E levels in 70% of thalassemic patients. Also in this study, after six

months of vitamins treatment, a significant improvement of MDA and GSH values and the activities of catalase and SOD were shown in patients of thalassemia major. The percentage of the number of patients who reached normal MDA and GSH values were 30% and 33% respectively and the activities of catalase and SOD improved in 20% and 24% of thalassemic patients respectively. After twelve months of treatment, a highly significant improvement of MDA and GSH values was observed. So the results were 63% and 83% of thalassemic patients who reached normal values of MDA and GSH respectively. Also in catalase and SOD activities the percentage of patients` number were 43% and 60% respectively who reached normal values. Under conditions of iron overload, increase of free radical production, peroxidative damages to tissues and depletion of endogenous antioxidants may be expected [21].

After six months of vitamins treatment, there was a significant improvement of the enzymes activities shown in thalassemia major patients. The percentage of the number of the patients who reached normal activities of ALT, AST, GPx, and GR were 33%, 30%, 27%, and 24% respectively. After twelve months of vitamins supplementation, there was a highly significant improvement of the enzymes activities, so the results of ALT, AST, GPx, and GR were 60%, 67%, 50%, and 57% respectively of the patients number were of normalized activities.

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 their organs from the damage resulting from iron overload which leads to more free radicals to cause organ dysfunction. Also, treatment with antioxidant vitamins improved the liver functions and reduced the percentage hemolysis of erythrocytes (results under publication, see reference 4) therefore improving the total Hb concentration.

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