INCREASED OXIDATIVE STRESS AND IRON OVERLOAD IN JORDANIAN β-THALASSEMIC CHILDREN

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□ β-Thalassemia (β-thal) is associated with abnormal synthesis of hemoglobin (Hb). Repeated blood transfusions in patients with β-thal major (β-TM) leads to an enhanced generation of reactive oxygen species (ROS), and subjects patients to peroxidative injury. We studied the antioxidant status and oxidative damage to children with β-thal in Jordan. Samples from 40 children with β-thal and 40 healthy controls were used. All children were under 13 years of age. Our results showed that plasma thiobarbituric acid reactive substances (TBARS) were elevated in β-thalassemic children compared to controls together with compensatory increase in superoxide dismutase (SOD) activity and decrease in catalase (CAT) activity. Elevated serum ferritin showed positive correlation with elevated liver enzyme levels except gamma glutamyl transferase (GGT), confirming liver involvement due to iron overload. Serum ferritin also showed a positive correlation with elevated TBARS and SOD, suggesting that iron overload is involved in the oxidative stress shown in cells.

Keywords Oxidative stress, Thalassemia, Glutathione (GSH), thiobarbituric acid reactive substances (TBARS)

INTRODUCTION

Thalassemias are a group of hereditary anemias which occur as a result of genetic disorders that affect the synthesis of normal hemoglobin (Hb), in which a reduced rate of synthesis of one or more of the globin chains leads
to defective Hb production, and damage to the red cells or their precursors (1). β-Thalassemia (β-thal) is more common in Mediterranean countries and islands including Cyprus, Sardinia, and Malta (2). However both α- and β-thal types are common in Africans and Black Americans (3). Thalassemia is usually associated with many complications such as hepatosplenomegaly, infections, gall stones and bone deformities that alter facial features and result in pathogenic fractures (4). Studies on β-thal patients suggest that they may develop symptoms of iron loading that includes chelating therapy complications, heart and liver diseases, and endocrinopathies (5).

Normally, erythrocytes degrade reactive oxygen species (ROS) via the actions of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (Gpx) (6). In β-thal major (β-TM), intraerythrocytic release of heme induces a glutathione (GSH)-dependent self-amplifying and self-propagating Hb oxidation pathway, resulting in injury to the thalassemic cell (6). In addition, iron can serve as a potent catalyst of lipid peroxidation (7). The presence of hypochromia may facilitate oxidation of the red cell membrane by reducing the amount of Hb available for buffering protection (8). The peripheral red cells of patients with β-TM demonstrate a variety of morphological, biochemical and metabolic changes, which specifically contribute to the extent and severity of lipid peroxidation and hemolysis (9). Unpaired α-Hb chains may denature and bind to the cell membrane, thus giving rise to cytoskeleton alterations and lipid peroxidation (10). The free α chains in β-thal increase autoxidation rates by about two times faster than normal Hb A (7). It has been reported that the accumulation and autoxidation of the unpaired α-globin chains in severe β-thal would generate ROS; superoxide (O2−) and hydrogen peroxide (H2O2) that would cause accelerated apoptosis and ineffective erythropoiesis (6,11).

Therefore, we hypothesize that oxidative stress is a major factor of morbidity in Jordanian β-thalassemic children and is correlated with iron overload and metabolic dysfunctions. In order to verify this hypothesis we evaluated different liver function enzymes such as aspartate amino transferase (AST), alanine amino transferase (ALT), gamma glutamyl transferase (GGT), alkaline phosphatase (ALP) and correlated them to ferritin levels in thalassemia patients receiving repeated blood transfusions. We also measured oxidative stress parameters including the lipid oxidation biomarker; malondialdehyde (MDA) and major antioxidant enzymes such as SOD and CAT in the serum of thalassemic patients and normal controls.

**MATERIALS AND METHODS**

**Patients and Controls**

Forty Jordanian children with β-TM who underwent periodical blood transfusions and received deferoxamine (DFO) as a chelating agent were
recruited for this study. Patients were free of hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus (HIV). This group was aged between 2 and 12 years old and was treated at the Thalassemia Unit at Princess Rahma Educational Hospital, Irbid, Jordan (20 female and 20 male patients). Another 40 healthy children aged between 5 and 12 years old was used as the control group (20 males and 20 females).

**Specimen Collection and Basic Analysis**

Blood samples were collected from an antecubital vein of β-TM patients and the control group. Blood was drawn in ethylene diamine tetracetic acid (EDTA), and plain tubes. All serum specimens (from patients and controls) were assayed directly for AST, ALT, GGT, ALP, enzymatic activity assays, as well as serum ferritin concentration. All serum specimens were separated and kept at −20°C until assayed for MDA, SOD, and CAT tests.

**Hemoglobin Analyses**

The Hb of whole blood was assayed using 50 μL anticoagulated blood in a blood analyzer (BC-2800 Mindray Auto Hematology Analyzer, Shenzhen, People’s Republic of China). The AST and ALT activity was assayed using commercial kits according to the manufacturer’s instructions (Teco Diagnostics, Annaheim, CA, USA). Absorbance readings were recorded using a Cecil 1010 Spectrophotometer (Cecil, Cambridge, Cambridgeshire, UK). The AST concentrations in IU/L was determined. Average absorbance per minute was multiplied by factor 1768. The GGT activity was assayed using a GGT kit according to the manufacturer’s instructions (Human, Wiesbaden, Germany). Concentration of GGT in IU/L was determined. Average absorbance per minute is multiplied by factor 1421. The ALP activity was assayed using a commercial kit according to the manufacturer’s instructions (Teco Diagnostics). Concentration of ALP in IU/L was determined. Average absorbance per minute was multiplied by factor 2187. Serum Ferritin concentration was assayed using a commercial kit (Bio Systems, Barcelona, Spain). The concentration of serum ferritin in μg/L was determined by the following formula: concentration of serum ferritin sample (μg/L) = (A2-A1) sample/(A2-A1) standard) * C where A1: first absorbance; A2: second absorbance; C standard: standard concentration.

The tissue thiobarbituric acid-reactive substances (TBARS) were measured according to the method of Ohkawa et al. (12). Briefly, 0.5 mL of serum was mixed with 0.5 mL of distilled water to bring the volume up to 1 mL, and an equal volume of 20% trichloroacetic acid (TCA) was added and incubated at 37°C for 20 min. and then centrifuged at 1,000 rpm. To 1 mL of TCA extract (the supernatant), 0.25 mL of 0.8% thiobarbituric acid (TBA) was added and heated in a water bath at 95°C for 1 hour until a faint
pink color appeared. After cooling, the color was extracted in 1 mL butanol and the absorbance was read at 532 nm. 1,1,3,3 Tetraethoxypropane (0–15 μmol/mL) was used as external standard.

Superoxide dismutase was assayed using a commercial kit (Cayman Chemical Company, Ann Arbor, MI, USA). Briefly, the assay buffer, sample buffer, radical buffer, and xanthine oxidase (XO) were prepared according to instructions provided by the manufacturing company. 0.01 mL of each standard and sample were added to the micro-titer plate. The reaction was initiated by adding 0.02 mL of diluted XO to all the wells, and then the plate was incubated on a shaker for 20 min. at room temperature. Absorbance at 405 nm was read using a plate reader. The concentration of SOD in U/mL was determined by using the following formula: SOD (U/mL) = [(sample linearized rate: y intercept/slope) * 0.23 mL/0.01 mL] * sample dilution.

Catalase was assayed using a commercial kit (Cayman Chemical Company). Briefly, the assay solutions (assay buffer, sample buffer, formaldehyde, CAT control, potassium hydroxide, H₂O₂, chromogen, potassium periodate and methanol) were prepared according to instructions provided by the manufacturing company. 0.02 mL of the standard or sample, 0.01 mL of assay buffer, 0.03 mL of methanol, and 0.02 mL of H₂O₂ were added to micro-titer plate wells. All wells were covered and incubated on a shaker for 20 min. at room temperature; the reaction was stopped by adding 0.03 mL potassium hydroxide to each well, followed by 0.03 mL chromogen, covering and incubating the plate on a shaker for another 10 min. at room temperature, then, 0.01 mL potassium periodate was added, and incubated for another 5 min. at room temperature. All wells are then read at 405 nm using a plate reader. The concentration of CAT in nmo/min./mL was determined according to the following formula: CAT activity = μM of sample/20 min. * Sample dilution.

Statistical Analyses
Data are presented as mean ± standard error of mean (SEM) using SPSS version 15. The results were analyzed by analysis of variance (ANOVA) followed by the unpaired Student’s t-test. A p value of <0.05 was considered significant. Correlations were calculated by Pearson’s correlation coefficients.

RESULTS
Hemoglobin Concentrations
Mean Hb concentration for all β-TM patients was significantly lower (p < 0.05) compared to the controls (9.1 ± 0.9 g/dL vs. 12.0 ± 0.9 g/dL, respectively). The mean Hb concentrations was higher for females than
Oxidative Stress and Pediatric Thalassemia in Jordan

All patients

All Controls

Female Patients

Male patients

Female Controls

Male Controls

FIGURE 1 Significant decreased in Hb concentrations in β-TM patients compared to healthy controls. Results are expressed as mean ± SEM (n = 40). *p < 0.05 was considered significant.

FIGURE 2 Increased serum ferritin in β-TM patients compared to healthy controls. All results shown are expressed as mean ± SEM (n = 40). *p < 0.05 was considered significant.

males (9.4 ± 0.8 g/dL vs. 8.7 ± 0.8 g/dL, respectively) (p > 0.05). Little difference in Hb concentrations was seen between healthy female and male controls (12.3 ± 0.9 g/dL and 11.9 ± 0.9 g/dL, respectively) (p > 0.05) (Figure 1).

Serum Ferritin Levels

A significant increase in serum ferritin concentration (p <0.05) in β-TM major patients compared to controls (2172 ± 941 μg/L vs. 59 ± 27 μg/L, respectively). No significant difference was seen between male and female individuals in patient and control groups (p >0.05) (Figure 2).

Liver Enzymes

A significant increase in the liver enzymes, AST and ALT, was seen in β-TM patients (p <0.05) compared to controls. The AST activity for β-TM patients was 61 ± 2.7 IU/L. The mean serum AST activity for the healthy individuals was 14 ± 2.2 IU/L (Figure 3). The ALT activity for β-TM patients was 76 ± 3.2 IU/L, whereas the mean serum ALT activity for the healthy individuals was 16 ± 0.8 IU/L (Figure 3). No significant different was seen in
GGT between patients and controls (11 ± 0.6 IU/L vs. 10 ± 0.5 IU/L, respectively) (p > 0.05) (Figure 3). No significant differences were seen between male and female patients and controls (data not shown) (p > 0.05). There was a positive correlation between serum ALT (p = 0.035, r = 0.334) and AST (p = 0.042, r = 0.324) concentrations and serum ferritin levels in β-TM patients compared to controls.

**Alkaline Phosphatase Activity**

The ALP reference range varies according to age subgroups. Therefore we divided our test individuals according to age as follows: 1–3 years old with reference range 281 IU/L, 4–6 years old with reference range up to 269 IU/L and 7–12 years old with reference range up to 300 IU/L.

For the 1–3 year old group, the β-TM patients mean ALP activity was 326 ± 145.7 IU/L (n = 5), while for the healthy individuals it was 196 ± 87.6 IU/L (p < 0.05). For the 4–6 year old group, the β-TM patients mean ALP activity was 253 ± 84 IU/L (n = 9), while for the healthy individuals it was 196 ± 65 IU/L (p < 0.05). For the 7–12 year old group, the β-TM patients mean ALP activity was 287 ± 56 IU/L (n = 26). The mean serum ALP activity for the healthy individuals was 196 ± 38 IU/L (p < 0.05). A significant increase in ALP was found when the mean concentration of ALP of all patients was compared to that of healthy controls (p < 0.05) (Figure 4).

**Lipid Peroxidation Measurements**

Malondialdehyde is formed as an end product of lipid peroxidation which reacts with TBA reagent under acidic conditions to generate a pink colored product. When the TBARS levels were measured for β-TM patients and controls, there was a significant increase in the TBARS in β-TM patients (3.8 ± 0.15 IU/L vs. 1.6 ± 0.04 IU/L) (p < 0.05) (Figure 5). There were no significant differences between male and female levels in both controls.
and β-TM patients (p > 0.05) (data not shown). A positive correlation (p = 0.044; r = 0.321) was found between TBARS generation and serum ferritin level in thalassemic patients compared to controls. However serum TBARS and ALT, AST levels in β-TM showed no significant correlation (p = 0.48, r = 0.088 and p = 0.59, r = 0.12, respectively).

**Superoxide Dismutase and Catalase Activity**

When SOD activity was measured in β-TM patients and controls, there was a significant increase in SOD activity in β-TM patients (210 ± 6.3 U/mL vs. 70 ± 3.1 U/mL) (p < 0.05) (Figure 6). No significant differences were found between male and female patients and controls (data not shown) (p > 0.05). The opposite picture was seen for CAT activity. A significant decrease in CAT activity was seen in β-TM patients when compared to controls (12.6 ± 3.1 nmol/min./mL vs. 59 ± 2.7 nmol/min./mL) (p < 0.05) (Figure 6). There was no significant difference between male and female levels in both controls and β-TM patients (p > 0.05) (data not shown). There was a statistically significant positive correlation between serum SOD and serum ferritin level in β-TM patients compared to controls (p = 0.046,
FIGURE 6 Serum SOD and CAT activity in β-TM patients and healthy controls. Results shown are expressed as mean ± SD (n = 40). *p < 0.05 was considered significant.

r = 0.32). In addition, a significant negative correlation between CAT generation and serum ferritin levels (p = 0.048, r = −0.31) was noted.

DISCUSSION

β-Thalassemias result from a number of underlying genetic defects that interfere with the synthesis of normal β chain (13). This results in a number of cellular defects that increase susceptibility to both endogenous and exogenous oxidants (14,15). The red blood cell (RBC) is unique among cells in that it contains very large concentrations of both iron (i.e., Hb) and oxygen. To prevent any complications of this dangerous combination, RBCs are normally protected by a number of endogenous antioxidants such as SOD, GSH, CAT, GSH/Gpx and membrane bound α-tocopherol (16–19). The Hb findings in β-TM and their control groups are shown in Figure 1. All β-TM patients’ Hb concentrations were significantly decreased compared to healthy controls (p < 0.05). This is expected in thalassemic patients, where normal Hb synthesis is impaired.

One complication of β-thal is iron overload (20,21). The serum ferritin of β-TM and the control groups are shown in Figure 2. All β-thal patients had significant increases in serum ferritin levels compared to the healthy individuals group (p <0.05). Ferritinemia was 37-fold more in β-TM patients compared to controls. This increase in serum ferritin indicates an existing iron overload in β-TM patients, probably due to multiple blood transfusions. It is estimated that with regular blood transfusion therapy, 0.32–0.64 mg/kg/day of iron is added to the body (20,21). Intestinal hyperabsorption in transfused patients makes the condition even worse (20,21). It was found that iron overload is the major cause of long-term complications of β-thal, including cardiac dysfunction, liver impairment, and endocrine dysfunction (20–22). The complications of iron overload, together with complications of the anemia, ineffective erythropoiesis and chelation therapy, are
major causes of morbidity and mortality in the transfusion-dependent β-thal patients (22). Indeed, iron intoxication can also lead to endocrinial complications such as hypogonadotrophin and hypoparathyroid (23,24).

Liver iron concentration exceeding the critical level may cause hepatic iron accumulation. This may cause an increase in hepatocellular injury and leakage of aminotransferase enzymes and ferritin to the plasma (5,25). Therefore, we assessed the extent of hepatocellular injury by determining the aminotransferases serum activity, which is mainly caused by leakage of the enzymes from cytoplasmic and mitochondrial compartments of injured hepatocytes to the plasma (26,27). The basic biochemical parameters of β-TM patients and the normal group are shown in Figures 3 and 4. Serum levels of AST, ALT, ALP but not GGT were significantly increased in β-TM patients as compared to controls (p <0.05). Similar results were reported by Livrea et al. (28).

Elevated levels of AST do not necessarily reflect a medical problem. The elevation in AST levels could result from strenuous physical exercise or other factors (29). In our study, we believe that the elevation in AST activity may be due to the iron overload seen in these patients. On the other hand, ALT is highly specific for hepatocellular damage, and its elevation could be due to liver damage secondary to iron overload (29). Our result is consistent with what has been found in different studies, where thalassemic patients showed increased liver enzymes (30–32). In our study, there was no significant increase in GGT activity (p >0.05). The GGT enzymatic activity is usually not tested alone; it may be tested in combination with ALP, to determine the kind of liver disease, whether it is of a biliary tract or bone origin. Our finding that ALP is high in thalassemic children could be due to higher physiological levels of ALP in normal children. It was reported that the increase in ALP activity in both normal and thalassemic children is mainly because all children have higher levels of ALP for their bone formation (33).

Iron may play a catalytic role in the initiation of free radical reactions, presumably through iron-initiated Fenton chemistry (34,35). The resulting ROS have the potential to damage cellular lipids, nucleic acids, proteins and carbohydrates. In animal models, high liver iron levels induce the elevation of lipid peroxides and oxidants (25,35). Increased lipid peroxidation markers have previously been observed in thalassemia patients (36,37). Dirican et al. (38), reported that thalassemic RBCs were more susceptible than normal cells to auto-oxidation. Plasma TBARS were studied as a marker of tissue injury and oxidative stress. Thiobarbituric acid reactive substances are well-recognized biomarker of lipid peroxidation (39,40). The serum TBARS findings in β-TM patients and their control groups are shown in Figure 5. As shown, serum levels of TBARS were significantly increased in β-TM as compared to controls (p <0.05). Our result is consistent with what has been found in different studies, where thalassemic patients show
increased TBARS (40–43). Regular blood transfusions and secondary iron overload make thalassemic erythrocytes prone to peroxidative injury (43). Our study shows that oxidative alterations to cell components can be shown in serum as a marked increase of MDA, reflected by increase of TBARS in plasma.

Since its clear from our data and other data that β-thal induces oxidative stress, the study of antioxidant enzymes could be very informative. Antioxidants in blood such as SOD and CAT significantly contribute to the total plasma antioxidant capacity (44), and are the first line of defense against oxidative stress (45). Superoxide dismutase catalyzes conversion of superoxide anion to H2O2 (46). As expected, our results show that serum levels of SOD were significantly increased in β-TM as compared to controls (p <0.05). This increase in SOD may be a compensatory mechanism to scavenge excess superoxide anion which resulted from oxidative stress (17,44). It has been reported by Meral et al. (7) that SOD levels of β-TM patients were elevated when compared to controls (45). On the other hand, when we studied CAT enzyme activity in both β-TM patients and controls, the enzyme activity was significantly decreased in β-TM patients as compared to controls (p <0.05). In fact, the loss of CAT activity impairs cellular protection against the endogenously generated H2O2, and increases iron mediated injury to the thalassemic erythrocyte membranes (47). The mechanism(s) underlying the decreased CAT activity was proposed by different investigators who suggested that nicotinamide adenine dinucleotide phosphate (NADPH) is important in maintaining CAT activity, and that the loss of NADPH would adversely affect erythrocyte enzyme activity (46–49). Catalase monomer contains a high affinity binding site for NADPH, which is crucial for the maintenance of CAT activity (49–51). β-Thalassemia major patients have demonstrated a significant decrease in NADPH (52,53). The loss of NADPH is most likely mediated by the release of heme and iron and a subsequent inhibition of other metabolic enzymes (54,55). This might explain our findings.

Our study suggests that in β-thal, the first target organ dysfunction is the liver, enhanced by an iron overload leading to a status of oxidative stress and initiates free radical reactions. Proper iron chelation and administration of adequate antioxidants may represent a promising way of counteracting oxidative stress and its deleterious effect on the prognosis of the disease.

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REFERENCES


52. Walter PB, Fung EB, Killilea DW, et al. Oxidative stress and inflammation in iron-overloaded...

