The Impact of Transfusion and Chelation on Oxidative Stress in Immigrant Syrian Children with β -Thalassemia

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Abstract Iron overload in β -thalassemia major and intermedia patients leads to oxidative stress and causes to formation of lipid hydroperoxides. Thiobarbituric acid reactive substances (TBARS) are a well established method for screening and monitoring of lipid peroxidation. We aimed to investigate serum TBARS and its relationship with biochemical and hematologic parameters of Turkish and immigrant Syrian β -thalassemia children reflecting the effects of this socioeconomic condition on follow up of these patients. Lipid peroxidation products (TBARS) of Turkish (TR) ($n = 62$, from the cities of Gaziantep and Sivas, Turkey) and Syrian (SYR) ($n = 34$, from Gaziantep, Turkey) β -thalassemia patients aged 2–17 years and 58 healthy subjects aged 2–16 years were studied. Liver and renal function tests, serum ferritin levels, white blood cell, absolute neutrophil and platelet counts, hemoglobin (Hb) levels of the patients were analyzed. Serum TBARS concentrations were found to be elevated in β -thalassemia patients compared to healthy subjects (mean: 12.47 ± 8.53 vs. 9.78 ± 7.09 , $p = 0.045$). In SYR patients mean pre-transfusional Hb level (7.26 ± 2.04 vs. 8.49 ± 1.01 ,

$p = 0.002$) was lower and ferritin levels (5983.56 ± 5065.56 vs. 3234.60 ± 2237.82 , $p = 0.001$), liver enzymes (ALT: 77.82 ± 76.48 vs. 42.13 ± 51.50 , $p = 0.005$) were higher when compared to TR group. Positive correlation between TBARS and ferritin levels ($p = 0.029$, $r = 0.231$) and liver enzymes (for ALT $p < 0.001$, $r = 0.373$) was observed. β -thalassemia patients are under more oxidative stress than healthy subjects. Liver is one of the major organs which are mainly affected by oxidative stress. War and migration might have caused inappropriate transfusion conditions and insufficient chelation therapy in the SYR group.

Keywords β -Thalassemia · TBARS · Transfusion

Introduction

Beta-thalassemia is a heterogeneous group of blood disorders characterized by decreased or absent synthesis of the beta-globin chain. Mutations of β -globin gene are located on the short arm of chromosome 11p15.5 leading to variable reduction of β -globin chain synthesis and relative α -globin chain excess resulting in intracellular participation of insoluble chains. Increased but ineffective erythropoiesis with many prematurely destroyed red cell precursors is related to α -globin chain excess. Iron overload caused by repeated blood transfusions is accompanied by increased iron absorption from intestine in β -thalassemia intermedia patients due to decreased hepcidin levels [1, 2]. This induces overproduction of reactive oxygen species (ROS) [3]. Iron-chelating compounds, desferrioxamine, deferiprone and deferasirox are used to counteract iron overload and improve life expectancy [4]. Non-heam ferrous iron is a strong pro-oxidant and is responsible for the production

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of damaging hydroxyl ($\cdot\text{OH}$) radical by Fenton reaction [5]. Non hem ferrous iron is one of the most important peroxidants in thalassemic patients and is responsible for production of damaging hydroxyl radical. Oxidative stress in the cellular environment results in the formation of highly reactive and unstable lipid hydroperoxides. Decomposition of the unstable peroxides derived from polyunsaturated fatty acids results in the formation of malondialdehyde (MDA), which can be quantified calorimetrically following its controlled reaction with thiobarbituric acid. The measurement of these “Thiobarbituric Acid Reactive Substances” (TBARS) is a well-established method for screening and monitoring lipid peroxidation [6, 7]. It has been shown that the level of plasma TBARS is increased in α and β -thalassemia patients [8–11].

The war in Syria has led to tremendous increase of the number of Syrian immigrant thalassemia patients during past 3 years in Gaziantep, a city in the southeastern part of Turkey. Blood transfusions and chelation therapy of these patients could not regularly be done in Syria because of the difficulties in access to sanitary services during the war. In this study we evaluated TBARS levels of Syrian and Turkish children with β -thalassemia major and intermedia that are followed at Gaziantep Children Hospital (Gaziantep, Turkey) and Cumhuriyet University Faculty of Medicine (Sivas, Turkey), Departments of Pediatric Hematology and Oncology. Total blood counts, liver and renal function tests, ferritin levels, status of splenectomy and hepatitis C positivity of the patients were also evaluated.

Materials and Methods

The study was approved by the local ethics committee of Gazi University Faculty of Medicine. All procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 2008. The participants were invited to attend voluntarily. Before admission, written informed consent was obtained from 58 healthy

volunteers, 90 β -thalassemia major and 6 β -thalassemia intermedia patients.

Study Population

Between June 2013 and December 2014, 62 Turkish (followed at Gaziantep Children Hospital, Gaziantep and Cumhuriyet University Faculty of Medicine, Sivas, Departments of Pediatric Hematology and Oncology) and 34 Syrian β -thalassemia (A total of 6 β -thalassemia intermedia and 90 β -thalassemia major) patients aged 2–17 years as patients group and 58 healthy volunteers (all had normal total blood count and hemoglobin electrophoresis) aged 2–16 years as control group were included in this study. Diagnoses of the thalassemia patients were confirmed by hemoglobin electrophoresis, blood count results and physical examination and genetic testing in selected patients. Patients' splenectomy status, anti HCV positivity, and medications that were used as iron chelating agents (deferasirox or defereprone) at the time of investigation were recorded in a chart which was prepared for this investigation (Fig. 1). Blood samples were collected at least 3–4 weeks after transfusion.

TBARS, High Performance Liquid Chromatography, Ferritin and Other Biochemical Parameters

The level of TBARS was analyzed to evaluate lipid peroxidation. Serum samples were obtained from all patients and control group. After centrifugation at 1000 rpm for 10 min TBARS were measured calorimetrically with ELISA (Enzyme-Linked Immunosorbent Assay) method by Cayman chemicals TBARS assay Kit (USA). The dynamic range of this assay is 1–50 μM for calorimetric method. Normal human serum contains 1.86–3.94 μM TBARS [6]. Blood urea nitrogen, creatinine, alanine aminotransferase (ALT), aspartate transaminase (AST),

Fig. 1 Distribution of patient group according to β -thalassemia type, splenectomy status, type of chelator drug and anti hepatitis C (HCV) positivity

Table 1 Demographical characteristics of patient groups

	Group TR (n = 62)	Group SYR (n = 34)	p value
Age (years)	8.0 ± 4.0 (2–17)	7.9 ± 4.3 (1–18)	0.855
Weight (kg)	23.3 ± 9.5 (10–55)	22.2 ± 7.5 (12–40)	0.739
Medication			0.028*
Deferasirox	44 (71.0%)	16 (47.1%)	
Deferiprone	18 (29.0%)	18 (52.9%)	
Thalassemia type			0.084
Major	56 (90.3%)	34 (100.0%)	
Intermedia	6 (9.7%)	–	
Splenectomy	23 (37.1%)	10 (29.4%)	0.395
HCV Positive	–	7 (20.60%)	<0.001*

TR Turkish, SYR Syrian, HCV Hepatitis C

*Statistically significant

uric acid levels were tested spectrophotometrically by Beckman coulter AU-5800 (USA). Hepatitis C (HCV) antibody was tested by ELISA method. Plasma ferritin level was evaluated by the method chemiluminescence with Beckman Coulter DXI-800 (USA). High performance liquid chromatography (HPLC) (Bio-rad, Variant 2) was used for diagnosis of β thalassemia. Total blood counts (white blood cell, hemoglobin, platelet, absolute neutrophil counts) were analyzed mindray BC-800 counter (China).

Statistical Analysis

All statistical analyses were performed by using SPSS 15.0 software (SPSS, Chicago, IL). Student's *t* test was used for normally distributed continuous variables. Categorical data were analyzed using Chi square and the Fisher exact test. Pearson correlation coefficient was used to determine the relation between TBARS and biochemical and hematological parameters. A *p* value <0.05 was considered to be statistically significant.

Results

We evaluated 96 β -thalassemia (6 intermedia and 90 major) patients including 52/96 (54.2%) male and 44/96 female (45.8%) also 58 healthy subjects (control group) that had 40/58 (69%) male and 18/58 (31.0%) female subjects. Mean age for thalassemia group was 7.97 ± 4.10 (median age = 7) and for control group was 8.12 ± 4.11 (median age = 7). There was no statistically significant difference as to age and gender between control and patient groups (*p* > 0.05). The patient group was sub grouped as Turkish (Group TR, n = 62) and Syrian (Group SYR, n = 34) patients. No difference according to age, weight, thalassemia type and splenectomy status was found

Fig. 2 **a** Levels of thiobarbituric acid reactive substances (TBARS) according to patient and control groups. **b** Levels of TBARS according to splenectomy. **c** Levels of TBARS according to chelating agent (medication)

between TR and SYR groups (Table 1). Deferasirox therapy was more frequent in Turkish patients than Syrian patients (*p* = 0.028). There were no HCV antibody positivity among Turkish patients (Table 1) when compared to Syrian patients (*p* = <0.001).

Mean TBARS level of patient group was $12.47 \pm 8.53 \mu\text{M}$ and control group was $9.78 \pm 7.09 \mu\text{M}$ (Fig. 2a). TBARS level of the patient group was found to be higher than control group (*p* = 0.045).

β -thalassemia major patients had higher levels of ferritin than thalassemia intermedia patients ($4435.61 \pm 3737.19 \text{ ng/ml}$ vs. $796.83 \pm 369.48 \text{ ng/ml}$). Because of the β thalassemia intermedia patients are so few we did not compare the results according to thalassemia types. When TR and SYR subgroups were compared (Table 2); ferritin, AST, ALT, BUN levels were higher in Syrian group than in Turkish group (*p* = 0.001, *p* = 0.001, *p* = 0.005, *p* = 0.007 respectively). Pretransfusion hemoglobin levels were lower in SYR group (*p* = 0.002). Hepatitis C antibody positivity was more frequent in SYR group (*p* = 0.001). Platelet counts were found to be higher among Turkish patients than Syrian patients (*p* = 0.042, Table 2). There was positive correlation between serum AST (*p* = 0.01, *r* = 0.429) and ALT (*p* = 0.01, *r* = 0.363) concentrations and serum ferritin levels.

The mean level of TBARS were not different according to splenectomy status and chelation type (*p* = 0.229, *p* = 0.180 respectively) (Fig. 2b, c).

After exclusion of HCV antibody positive patients (n = 7) correlation analysis was repeated among TBARS levels and biochemical and hematologic parameters of the 89 β -thalassemia patients (SYR and TR group without anti

HCV positivity) There was positive correlation between TBARS and, AST, ALT, ferritin levels, WBC and ANC values (Table 3).

Discussion

Transfusional iron overload is the major cause of hemosiderosis in beta thalassemia major patients. In thalassemia intermedia patients enhanced iron absorption from intestines further increases iron overload [1]. Iron-chelating agents such as deferoxamine, deferiprone, deferasirox are frequently used for iron chelation. Regular chelation is crucial for the prevention of complications of iron overload [1, 4]. In our study, SYR group had lower mean pretransfusional Hb level and higher mean ferritin, AST, ALT and BUN levels when compared to TR group (Table 2). These findings may be attributed to irregular transfusion and chelation of SYR group due to war and migration. HCV antibody positivity in SYR group might have been caused by inadequate transfusion facilities in Syria (Table 2).

Regular red blood cell transfusions, erythrocyte hyperhemolysis and increased iron absorption are the major causes of iron overload in thalassemia patients. Thalassemia intermedia patients need less transfusion than thalassemia major patients [12]. In our study, as we expected β -thalassemia major patients had higher levels of ferritin than thalassemia intermedia patients (4435.61 ± 3737.19 vs. 796.83 ± 369.48 ng/ml). But we could not statistically compare these two subtypes because the intermedia patients are so few.

Non-heme ferrous iron is a strong pro-oxidant and is responsible for the production of damaging hydroxyl ($\cdot\text{OH}$) radical by Fenton reaction [5]. Increased oxygen-free radicals cause lipid peroxidation. Thiobarbituric acid reactive substances are good indicators of lipid peroxidation. Recently some investigations showed TBARS are increased in β -thalassemia major patients [9, 10, 13, 14]. In our study we found higher mean levels of TBARS in β -thalassemia patients when compared to the control group in accordance with the previous studies (Fig. 1a). This finding showed thalassemia patients are under more oxidative

Table 2 Evaluation of laboratory parameters in Turkish (TR) and Syrian (SYR) subgroups

	Group TR n = 62 (mean $\pm$ SD)	Group SYR n = 34 (mean $\pm$ SD)	p
TBARS (μM)	11.67 ± 8.08 (1.12–40.4)	13.93 ± 9.24 (1.12–44.68)	0.220
HCV Antibody positivity (n)	0/62 (0%)	7/34 (20.6%)	<0.001*
Ferritin (ng/ml)	3234.60 ± 2237.82 (165–9620)	5983.56 ± 5065.56 (369–28669)	0.001*
AST (U/L)	41.15 ± 26.70 (19–211)	70.88 ± 51.75 (18–273)	0.001*
ALT (U/L)	42.13 ± 51.50 (9–377)	77.82 ± 76.48 (12–291)	0.005*
BUN (mg/dl)	27.61 ± 6.99 (13–49)	23.03 ± 6.70 (9–36)	0.020*
Uric acid	3.78 ± 1.07 (1–7)	4.22 ± 1.13 (2–7)	0.220
Hb (g/L)	84.9 ± 10.1 (60–100)	72.6 ± 20.4 (30–100)	0.002*
WBC ($\times 10^9/\text{L}$)	14.275 ± 9.241 (4.40–49.80)	13.454 ± 11.761 (2.73–56.50)	0.137
ANC ($\times 10^9/\text{L}$)	7.919 ± 6.069 (1.60–34.00)	6.767 ± 6.491 (1.31–30.00)	0.070
Platelets ($\times 10^9/\text{L}$)	465 ± 224 (148–1294)	390 ± 250 (50–1043)	0.042*

SD standard deviation, TBARS thiobarbituric acid reactive substances, HCV hepatitis C, ALT alanine aminotransferase, AST aspartate transaminase, BUN blood urea nitrogen, Hb hemoglobin, WBC white blood cell, ANC absolute neutrophil count

*Statistically significant

Table 3 Correlation analysis between TBARS and biochemical and hematological parameters of the patient group (HCV antibody negative Syrian and Turkish β -thalassemia patients, n = 89)

	Ferritin	AST	ALT	BUN	UA	WBC	ANC	PLT	Hb
TBARS									
r									
0.231*		0.415**	0.373**	0.115	0.111	0.266*	0.237*	0.184	0.043
p									
0.029		<0.001	<0.001	0.285	0.301	0.012	0.025	0.084	0.686

ALT alanine aminotransferase, AST aspartate transaminase, BUN blood urea nitrogen, UA uric acid, Hb hemoglobin, WBC white blood cell, ANC absolute neutrophil count

* Correlation is significant at the 0.05 level (2-tailed)

** Correlation is significant at the 0.01 level (2-tailed)

stress than healthy subjects. The levels of TBARS were not different according to type of chelator drug and splenectomy status (Fig. 2b, c). During our literature search we did not find any investigation which analyzes the relation between chelating agents and TBARS.

In our study, among HCV negative β -thalassemia patients there was positive correlation between mean ferritin and TBARS levels. Also, we found positive correlation between serum AST and ALT concentrations and TBARS levels among HCV negative β -thalassemia patients (Table 3). In a study by Abdalla et al. [15], positive correlation between TBARS and serum ferritin levels was found however no correlation was shown between TBARS and serum AST and ALT concentrations. In β -thalassemia patients the first target organ that is affected by diminished antioxidant reserves is the liver, enhanced by an iron overload leading to an acute free radicals action [14]. Increased transaminasaemia is caused by cytolysis and hepatic necroinflammatory mechanisms. In the liver, lipid peroxidation is associated with impairment of membrane-dependent functions of mitochondria and lysosomes [16]. In accordance with these pathogenic mechanisms; increased ferritin, AST and ALT levels and their positive correlation with TBARS, may be attributed to increased oxidative stress caused by iron overload leading to increased liver enzymes in beta thalassemia patients. In a study done by Dasgupta et al. [17] it was found that TBARS levels showed significant correlation with serum uric acid, ferritin and iron levels. However in our study there was no correlation between TBARS and uric acid levels (Table 3).

In our study there are some limitations that we could not research the genetic modifiers of phenotype, economic conditions, nutritional status of the Turkish and Syrian β -thalassemia patients. These should be searched in further studies with larger series.

Conclusion

The present study did not only evaluate the TBARS levels of different ethnical groups but also highlighted the status of transfusion and chelation of immigrant patients showing decreased pretransfusion hemoglobin levels and increased mean ferritin levels in SYR group that lack sufficient medical care when compared to TR group. Also we showed β -thalassemia patients had more oxidative stress than normal population and liver is one of the most important organs that are mainly affected from oxidative stress. The relation between TBARS and chelation therapy should be investigated with further studies.

Compliance with Ethical Standards

Conflict of interest There is no conflict of interest.

Author Contributors MKC, BB, FB and EÜK: Collected data analyzed data and wrote the manuscript; ÖGB: Analyzed biochemical and hematologic parameters. ÜK: Involved in review of literature, data collection and drafting the manuscript. All authors have read and approved the final manuscript. MKC will act as guarantor for this paper.

Human and Animal Rights The study was approved by the local ethics committee of Gazi University Faculty of Medicine. All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The participants were invited to attend voluntarily. Before admission, written informed consent was obtained from 58 healthy volunteers, 90 β -thalassemia major and 6 β -thalassemia intermedia patients.

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