

Detection of some antioxidant markers in saliva of patients with beta thalassemia major

Iyden Kamil Mohammed*

* University of Baghdad /Al-Khwarizmi College of Eng. /Biomedical Eng. Department
aydenel_1969@yahoo.com

Abstract

Background : Although evaluation and maintenance of antioxidant defence can be useful in protecting β -thalassemia patients from more serious complications of the disease, there are limited studies about assessment of antioxidant capacity in beta-thalassemic patients particularly in saliva.

Methodology:Thirty patients with β thalassemia major were involved in this study in thalassemia center / Ebn- Albalady hospital in Baghdad, and fifteen normal subjects with matched age and sex were also involved and considered as control group. Age, gender, blood groups, BMI, secretory status, and antioxidant markers were determined in the saliva of normal and diseased subjects, however the status of HCV infection, liver and spleen are evaluated in beta-thalassemic patients.

Results: Frequency of non-secretors patients (43.3%) is significantly higher than those in control group (20%), while there is no significant difference in the distribution of ABO blood groups among subjects in both groups. Serum ferritin is significantly elevated from (34 ± 4.4 ng/ml) in control group up to (4442 ± 875 ng/ml) in patients, while the level of all antioxidant markers in the saliva reveals non-significant differences between patients and control group except albumin marker which is significantly dropped in the saliva of patients in comparison with control. Ferritin is negatively correlated with hepatomegaly ($r = -0.169$), splenectomized ($r = -0.199$), HCV-infected ($r = -0.095$), and obese ($r = -0.159$) patients. On the contrary, albumin shows positively association with all these pathologic condition, and its level is non-significantly increased in patients with hepatomegaly ($r = 0.031$), splenectomy ($r = 0.177$), HCV infection ($r = 0.133$), and obesity ($r = 0.170$).

Conclusion:Non-secretory trait may participate in exacerbation the severity of complication in TM patients, and salivary level of albumin marker may be serve as a predictive value that reflect the antioxidant status.

Key words: beta-thalassemia major, secretory status, ferritin, antioxidants, salivary biomarkers, albumin, splenectomy

Introduction

Beta thalassemia is an autosomal hematological disorder that is the result of genetically deficient synthesis of the beta-globin chains of hemoglobin⁽¹⁾.The total annual incidence of symptomatic individuals is estimated at 1 in 100,000 throughout the world and Iraq is one of the countries with a relatively high frequency of beta-thalassemia specifically in the northern part due to the high rate of consanguineous marriages in this region⁽²⁻⁴⁾.Thalassemia major presents as a progressive anemia during 6 to 24 months of age that produce severe life-threatening anemia(Hb 2-7 g/dl), fatigue, dyspnea, poor appetite, hepatosplenomegaly, heart failure and bone deformation, delayed puberty, and in some patients, death would result without chronic blood transfusions⁽⁵⁻⁹⁾. Therefore, life-long regular transfusion is require and started when a patient is unable to maintain Hb > 7 g/dL or if there is poor growth^(10, 11).However, iron overload can result in multiple progressive oxidative damage in

different organs, serious infections (e.g. hepatitis B or C), hypersensitivity reactions, cholecystitis, leg ulcers, and fractures^(9, 12-15). Therefore, blood transfusion and iron chelation therapy have improved the quality of life and life-span to an age of around 30 years⁽¹⁶⁻¹⁸⁾. Splenectomy is another treatment option to reduce blood consumption and decrease iron overload-related organ damage, but Splenectomised patients are at as much as 30 times a higher risk of developing sepsis than the general population⁽¹⁹⁻²¹⁾. Accordingly, evaluation and maintenance of antioxidant defence can be useful in protecting β -thalassemia patients from more serious complications of the disease^(22, 23). There are limited studies about assessment of antioxidant capacity in thalassemic patients, some of these studies focused on total antioxidant capacity (TAC)^(13,24,25). However, other researchers investigated trace elements (e.g. calcium, phosphorus, bicarbonate, potassium, and sodium), enzymes (e.g. peroxidase, LDH, lysozymes, AST and ALT), and metabolites (urea, uric acid, glutathione, and malondialdehyde) in saliva of thalassemia patients⁽²⁶⁻³³⁾.

The present study is conducted to detect different intrinsic antioxidant in the saliva of major thalassemic patients which include (total antioxidant capacity, reduced glutathione (GSH), Albumin, uric acid, and urea) and their relation with the status of liver, spleen, hepatitis-C infection (HCV), and body mass index (BMI) categories.

Materials and Methods

Thirty patients with β thalassemia major were recruited in this study from thalassemia center / Ebn- Albalady hospital/ Baghdad/ Iraq, and fifteen normal subjects with matched age and sex were also involved and considered as control group. All patients were blood transfusion-dependent, and on iron chelation therapy. By the consent of specialist, medical history information from patient's file were recorded including; sex, age, blood groups, blood transfusion rate, and complications in liver, spleen, and hepatitis C virus (HCV) infection.

The weight and height of all subjects in patients and control groups were measured to calculate body mass index BMI. For adults (> 20 years aged), a BMI of less than 18.5 is considered underweight, while a BMI greater than 25 is considered overweight and above 30 is considered obese⁽³⁴⁾. However, for children and adolescents (2-20 years aged), a BMI that is less than the 5th percentile is considered underweight and above the 95th percentile is considered obese, while those with a BMI between the 85th and 95th percentile are considered to be overweight⁽³⁵⁾.

Blood and saliva samples were collected from all subjects (normal and TM patients). About 3-5 ml venous peripheral blood were aspirated from antecubital vein at morning in a plane tube (without anticoagulant), and left for 15 minutes at 4°C to clot, then centrifuged at 3000 rpm for 10 minutes to collect serum to be used for determination of ferritin marker by using an automated quantitative Mini VIDAS test from (Vidas ® Ferritin, BioMerieux ® Lyon- France). However, about 3-5 ml of saliva was collected from all subjects after rinsing their mouth 2 times with cold drinking water 5-10 minutes prior to collection, then transferred into sterile container. All samples centrifuged at 3000 rpm for 10 minutes to eliminate any debris. The supernatant was divided into 2 aliquots; the first one was placed in a bath of boiling water for 10-15 minutes and centrifuged at 3000 rpm for 2 minutes then the supernatant aspirated and directly used to determine secretory status based on hemagglutination inhibition test by using diluted blood grouping reagent Anti-A, Anti-B (Biotec, Germany), or Anti-H lectin (Biorex, UK)⁽³⁶⁾. The second aliquot of saliva was used in determination of five biochemical markers which include urea, uric

acid, albumin, glutathione (GSH), and total antioxidants (TAO). The concentration of urea, uric acid, and albumin in saliva are estimated by using a specific kit from (AGAPPE diagnostics-Switzerland, GmbH) based on colorimetric method⁽³⁷⁻³⁹⁾. However, the concentration of GSH was determined by using competitive-ELISA kit provided from (Elabscience Biotechnology Co, Ltd, China), while TAO level was determined by using colorimetric detection kit provided from (Elabscience Biotechnology Co, Ltd, China).

Values were reported either as mean $\pm$ standard deviation ($M \pm SD$), mean $\pm$ standard error ($M \pm SE$) or percentage (%). Statistical analyses were carried out by using Vassar Stats Web Site for Statistical Computation based on Student t-test, one-way analysis of variance test (ANOVA), chi square test, and Pearson's correlation (r) test. Significance of all statistical tests were 2-tailed, and a P value of < 0.05 was considered as statistically significant.

Results

The results show non-significant difference in the mean of age and frequency of gender between patients and control groups which are nearly matched (Table-1). However, the BMI of patients ($17.2 \pm 3.05 \text{ kg/m}^2$) is significantly lower than that of control ($23.2 \pm 5.55 \text{ kg/m}^2$), also the frequency of underweight category in patients (16.7%) is higher than that of normal subjects in control group (6.7%) but without significant association (Table-2). Concerning with the distribution of secretory status and ABO blood groups, the results show that the frequency of non-secretors patients (43.3%) is significantly higher than those in control group (20%), while there is no significant difference in the distribution of ABO blood groups among subjects in both groups (Table-3).

On the other hand, serum ferritin is significantly elevated from ($34 \pm 4.4 \text{ ng/ml}$) in control group up to ($4442 \pm 875 \text{ ng/ml}$) in patients, while the level of all antioxidant markers in the saliva reveal non-significant differences between patients and control group except albumin marker which is significantly dropped in the saliva of patients down to ($0.92 \pm 0.02 \text{ g/dl}$) in comparison with ($1.01 \pm 0.01 \text{ g/dl}$) in control (Table-4). Furthermore, characterization of pathologic conditions in liver, spleen, and HCV infection in beta-thalassemic patients shows that 70% of them are presented with hepatomegaly, 56.7% with splenomegaly, 23.3% splenectomized, and 20% are infected with HCV (Table-5). Statistical analysis of correlation between these pathologic condition versus ferritin and albumin levels, as antioxidant markers, the result shows that ferritin is negatively correlated with these conditions and its level is non-significantly decreased in hepatomegaly ($r = -0.169$), splenectomized ($r = -0.199$), HCV-infected ($r = -0.095$), and obese ($r = -0.159$) patients (Table-6). In contrary, albumin shows positively association with all these pathologic condition, and its level is non-significantly increased in patients with hepatomegaly ($r = 0.031$), splenectomy ($r = 0.177$), HCV infection ($r = 0.133$), and obesity ($r = 0.170$).

Table-1: Characterization of healthy and patient groups

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Character		Control (N=15)	Patients (N=30)	Significance
Age (M±SD) years		16.2 ± 5.4	13.4 ± 6.3	[NS]
Gender (n) (%)	Male	8 (53.3%)	15 (50%)	[NS]
	Female	7 (46.7%)	15 (50%)	
[NS]: non-significant difference				

Table-2: Determination of BMI categories in control and patients groups

Table 2: Determination of BMI categories in control and patients groups				
BMI (kg/m ²)		Control (N=15)	Patients (N=30)	Significance
M±SD		23.2 ± 5.55	17.2 ± 3.05	[S]
BMI category (n) (%)	Under weight	1 (6.7%)	5 (16.7%)	[NS]
	Normal	7 (46.7%)	20 (66.7%)	
	Overweight	2 (13.3%)	1 (3.3%)	
	Obese	5 (33.3%)	4 (13.3%)	
[S]:significant difference; [NS]: non-significant difference				

Table-3: Distribution of blood groups and secretory status in control and patients groups

Frequency		Control (N=15)	Patients (N=30)	Significance
Secretory status (n) (%)	Secretors	12 (80%)	17 (56.7%)	[S]
	Non-secretors	3 (20%)	13 (43.3%)	
Blood groups (n) (%)	A	7 (46.7%)	9 (30%)	[NS]
	B	4 (26.7%)	8 (26.7%)	
	AB	1 (6.6%)	1 (3.3%)	
	O	3 (20%)	12 (40%)	
[S]:significant difference; [NS]: non-significant difference				

Table-4: Concentration of antioxidant markers in the saliva of control and patients groups

Marker	Concentration (M±SE)		Significance
	Control (N=15)	Patients (N=30)	
Ferritin*(ng/ml)	34 ± 4.4	4442 ± 875	P= 0.0009
GSH (µg/ml)	12.2 ± 1.04	12.7 ± 0.83	P= 0.753
TAO (U/ml)	3.4 ± 0.40	4.1 ± 0.38	P= 0.313
Urea (mg/dl)	24.0 ± 1.3	24.3 ± 1.4	P= 0.888
Uric Acid (mg/dl)	4.7 ± 0.26	5.1 ± 0.43	P= 0.580
Albumin (g/dl)	1.01 ± 0.01	0.92 ± 0.02	P= 0.029
(*): only ferritin detected in serum			

Table-5: The status of liver, spleen, and HCV infection in patients group

Status of	Categories	Cases number	Cases %
Liver	Normal	9	30%
	Hepatomegaly	21	70%
Spleen	Normal	6	20%
	Splenomegaly	17	56.7%
	Splenectomy	7	23.3%
HCV infection	Negative	24	80%
	Positive	6	20%

Table-6: Correlation of ferritin and albumin levels with pathologic conditions in patients

Correlation (r) of	Versus	
	Ferritin	Albumin
Hepatomegaly	r= - 0.169 [NS]	r= 0.031 [NS]
Splenectomy	r= - 0.199 [NS]	r= 0.177 [NS]
Positive HCV infection	r= - 0.095 [NS]	r= 0.133 [NS]
Obesity	r= - 0.159 [NS]	r= 0.170 [NS]

Discussion

The result of this study showed significant difference in the BMI between the control and patients and about (16.7%) of the patients have underweight body stature in comparison to (6.7%) of control group (Table-2). Several other studies also reported high frequency of BMI **below the 3rd percentile depending on** the age of TM patients, it has been found that 12.4% of TM patients under 10 years have underweight BMI up to 46.5% in patients with age above 10 years⁽⁴⁰⁻⁴³⁾. The most important etiologies of this result are possibly the presence of multiple endocrinopathies, under-nutrition as well as other complications of thalassemia such as tissue hypoxia, and side effects of chelating therapy with desferrioxamine^(40, 44-46). Also it may be due to iron overload resulting in multiple progressive organ damage which includes growth retardation and delay of sexual maturation in children, and later involvement of the heart, liver, and endocrine system^(2, 47).

On the other hand, it has been reported that secretor and non-secretor status as well as certain blood group makes somebody prone to communicable and non-communicable diseases^(48, 49). Accordingly, this study demonstrated non-significant difference in the distribution of blood groups between the control and patients, although 'O' group was the most frequent (40%) among TM patients (Table-3) which is comparable with previous result obtained by Adaay *et al.*, (2011) who found that blood group O is the highest frequent (42.8%) among TM patients⁽⁵⁰⁾, although its normal distribution in Iraqi people constitute about 34.7%⁽⁵¹⁾. Moreover, the results of this study reported high significant frequency of non-secretors (43.3%) in thalassemic patients group in comparison with those in control group (20%) as shown in (Table-3). It is generally known that about 80% of the world's population are secretors of ABH antigens and only 20% are non-secretors but with some racial differences⁽⁵²⁾. Therefore, determining ABH secretor phenotype may be useful as risk factor determinates for a number of conditions because there are certain diseases which

show higher incidence association with non-secretors including heart disease, diabetes, insulin resistance, certain types of cancer, autoimmune diseases, and others⁽⁵³⁻⁵⁵⁾.

In respect to serum ferritin which is a major iron storage protein of liver, spleen, bone marrow and other tissue of the body⁽⁸⁾, our result recorded high ferritin level (4442 ± 875 ng/ml) in the serum of TM patients in comparison with (34 ± 4.4 ng/ml) in normal subjects (Table-4). Comparable to other studies, it has been found that highly elevation of **serum ferritin level** increase the risk of development impaired glucose tolerance in frequently transfused TM patients particularly those under poor compliance with iron chelation therapy^(42, 56-57). **Since** the relationship of ferritin level (as parameter of iron overload) and different complications in TM patients was the main objective of several studies, our study found that ferritin is negatively correlated with hepatomegaly, splenectomized, HCV-infected, and obese TM patients, and its level is non-significantly decreased in these conditions (Table-6). Several studies suggested that both serum level of ferritin (as parameter of iron overload) and bilirubin (as parameter of jaundice) were significantly raised in thalassemic patients, but no statistical correlation was found between these two parameters⁽⁵⁸⁾. Also Mansi and Aburjai, (2008) suggested that high ferritin level may affect lipids pattern among patients with beta-thalassemia major⁽⁵⁹⁾. However, Amile *et al.*, (2008) stated that both HCV infection and iron overload are the main causes of abnormal liver function in patients with thalassemia⁽⁶⁰⁾. Subsequently, it has been reported that serum ferritin level was highly significant in HCV positive thalassemic patients in comparison with HCV negative⁽⁶¹⁻⁶³⁾. Moreover, the prevalence of HCV infection was increased with increasing age of the patients, and this could be explain by increase chance of exposure to infected blood or by increased frequency of admission to hospital with increase possibility to exposure to infected device or material^(64, 65). In contrast, laboratory finding in both splenectomized and non-splenectomized TM patients showed non-significant differences in ferritin level^(43, 66).

Concerning with salivary levels of antioxidant markers, our results found that all antioxidants markers involved in this study were detectable in saliva, but with non-significant differences between patients and control group except albumin marker which is significantly dropped in the saliva of patients down to (0.92 ± 0.02 g/dl) in comparison with (1.01 ± 0.01 g/dl) in control (Table-4). Since saliva contains constituents of non salivary origin and serum-derived components, therefore, saliva has been proposed to be a good surrogate of blood for diagnostic purposes^(67, 68).

There are limited studies about assessment of antioxidant capacity in thalassemic patients whether in serum or in saliva. Some of these studies reported no significant differences or depletion of antioxidants in thalassemic patients^(13, 24-25). Recently, Tolba *et al.*, (2015) in study on Egyptian TM patients found decreased antioxidants level in their serum which contributing to the development of atherosclerosis in TM patients⁽⁶⁹⁾. However, other studies reported that endogenous antioxidants such as ferritin, uric acid (UA) and bilirubin can result in increased level of TAC in the patients with Beta-thalassemia major^(70, 71), and this elevation in their levels is a compensatory response arising from excessive oxidative stress that may be relevant to chelation therapy by deferoxamine^(72, 73). Recent study found an increase in serum and saliva uric acid in patients with beta thalassemia major, that may be due to counteract the increased oxidative stress resulted from increased serum and saliva level of malondialdehyde biomarker⁽³³⁾.

In contrast to our results, it was found that albumin level in TM patients showed non-significant difference from normal subjects, also no significant correlation with Hb among beta-thalassemic patients^(74, 75). However, our result showed decreasing in its salivary level (Table-4), this decreasing may be due to converting normal albumin into ischemia modified albumin-IMA as a result of ischemic events that is currently used as an early marker for myocardial ischemia and acute coronary syndrome in conditions other than ischemic heart and has been suggested that elevated levels of IMA may reflect a generalized rather than organ- or tissue-specific state of oxidative stress^(76, 77), because recent study suggested that increased levels of IMA in thalassemic patients are likely to be a result of iron-induced oxidative stress and hence it's potential significance as a new marker of oxidative stress in such patients.

Conclusion

The findings obtained from this study particularly salivary level of albumin marker may be serve as a predictive value that reflect the antioxidant status in the TM patient and can be used for monitoring prognosis of complications in such patients. Furthermore, non-secretory trait may participate in exacerbation the severity of complication in TM patients. Therefore, searching for further markers in the saliva of TM patients is the best choice to monitor the severity of oxidative stress in their body not for diagnostic purposes, but to improve their antioxidant status as soon as possible via administration of natural antioxidant agents either as supplements or by enhancing nutritional status.

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