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Original Article

Comparison of Levels of Iron, Ferritin and Aminotransferases in Serum and Saliva of Beta Thalassemia Major Patients

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ABSTRACT

Background: *β - thalassemia* major is inherited disorder of single gene defect that occur as a result of more than 200 mutations in the beta goblin gene causing its impaired synthesis. Frequent blood transfusions are necessary for the treatment and management of thalassemia major patients. These excessive transfusions can often lead to iron overload that can damage many organs including liver, endocrine glands and spleen leading to life-threatening complications. This study was conducted to estimate the variables like iron ferritin and aminotransferases (ALT, AST) in serum and saliva of β-thalassemia patients.

Materials and methods: This study was conducted during a year from January-December 2018. It was a cross sectional comparative study. Forty diagnosed cases of β-thalassemia major were included in the study. From each patient both serum and saliva samples were collected. The levels of ALT and AST in serum and saliva samples of all study subjects were measured on chemistry analyzer using enzymatic kinetic method. Iron levels were measured by using ferrozine chromogenic method and absorbance were taken on microplate reader (Bio-RAD analyzer) capability and serum and saliva ferritin was measured by enzyme-linked immune-sorbent assay (ELISA) method.

Results: The results of the study showed a statistically significant difference between these two groups whereas the correlation analysis showed moderate correlation between serum and saliva iron and AST levels, weak correlation between serum and saliva ferritin level while strong correlation between serum and saliva ALT level.

Conclusion: This study concludes that saliva cannot be used as diagnostic marker for iron overload and liver damage in β -thalassemia major patients however saliva can be used as predictive marker.

Keywords: β – thalassemia, Saliva, Serum, Aminotransferases, blood transfusion, Iron Overload, Hepatic Damage

Introduction

Thalassemia is an inherited disorder of hemoglobin (Hb) synthesis [(1)]. It is one of the common single gene disorders worldwide, affecting both men and women equally. It results in moderate to serious health issue and poses the economic burden to patients at risk [(2)]. Thalassemia has been prevalent in all over the world due to intermarriages among various ethnic groups. About 80 to 90 million of the world population are carriers of this life threatening disorder [(3).]

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β -thalassemia is inherited disorder of single gene defect that occur as a result of more than 200 mutations in the (HBB) beta globin gene [(4)]. In Thalassemia the concentration of (α) alpha globin chains become relatively in excess in unbound form due to either absence (β^0) or reduced amount (β^+) of beta goblin chains. The excess amount of alpha chain results in precipitation within erythroid precursors and within the bone marrow that causes immature cell death and thus leads to unsuccessful erythropoiesis [(5)].

According to WHO report which estimates that per year 5000-9000 children are born with β- thalassemia in Pakistan and 9.8 million carriers that forms 5 to 7% of the total population [(6)]. According to Thalassemia Federation of Pakistan the prevalence rate of β-Thalassemia in Pakistani population is about 6%. There are more than 50,000 thalassemia patient

registered with associations and treatment centers all over the country [7].

In β Thalassemia major the affected Individuals are unable to make enough healthy red blood cells that is why they are totally dependent on blood transfusion throughout their life. This condition begins with anemia and patients need regular leucoreduced packed red blood cells every 4-6 week [8]. Various complications caused by this disease including growth retardation, endocrine dysfunction, hypothyroidism, progressive liver failure and abnormal kidney function [9]. Under normal circumstances liver contains around one third of body iron that is stored in ferritin and hemosiderin form. Hepatocytes constitute about 98% of hepatic iron and remaining iron about 1.5-2% present in, bile ductular cells, reticuloendothelial cells and fibroblast. Therefore, liver plays a key role in iron homeostasis. Iron that exceeds the required amount within the cell accumulates in iron stores in the form of ferritin and hemosiderin. Continued accumulation of iron in cells leads to cellular toxicity [10].

In thalassemic patients when the concentration of hemoglobin and supply of oxygen is reduced the red cell concentrate is administered which improves the oxygen carrying capacity and eventually increases supply of oxygen to body cells [11]. Blood transfusion of 500 mL constitutes about 250 mg iron and these patients need regular leucoreduced pack red blood cells every 4-6 week and thus iron overload is unpreventable that can be treated therapeutically by giving iron chelation [12].

Among the different complications as described above, iron overload is the major complication that needs attention. Although, blood transfusions are inevitable for thalassemia patients, progressive transfusions necessarily result in iron overload as in humans excess iron cannot be managed to remove actively [13]. The iron overload in the body can be determined with the help of ferritin, iron, non - transferrin bound iron (NTBI) and TIBC levels in serum. Regular monitoring of excess iron and iron chelation effects play role in effective management in thalassemia [14]. The common method of *Beta Thalassemia* major evaluation is through serum ferritin levels estimation that directly relates to iron overload in the body. Ferritin is the most frequently used measure as it is inexpensive, widely available, and reliable, with extensive clinical validation in monitoring iron status [15].

In recent years saliva is used as diagnostic fluid in which different parameters are being measured includes aminotransferases (ALT, AST), iron and ferritin. Canatan and Akdeniz, in 2012 studied iron and ferritin level in serum and saliva of thalassemia and iron deficiency anemia patients [16].

Shivashankara and his colleagues inducted 50 alcoholics' patients in their study and analyzed their liver enzymes level. They found that serum and salivary levels of aminotransferases were high in alcoholics then in control group [17]. Jagannathan and his coworker studied ferritin level in saliva and serum of iron deficiency anemia patients. The results of the study showed markedly increased salivary ferritin levels in comparison to serum ferritin levels in iron deficiency anemia patients .The mean salivary ferritin value of subjects with iron deficiency anemia was 153.24 ± 26.58 µg/dl and in control subject was 93.87 ± 23.15 µg/dl as compared to a serum ferritin levels of normal subjects was 82.34 ± 9.6 µg/dl in and in iron deficient subjects 31.69 ± 6.28 µg/dl [18].

Whole saliva, however, is most frequently used for diagnosis of systemic diseases. The constituents of saliva are derived from the local vasculature of the salivary glands and also reach the oral cavity via the flow of gingival fluid. Analysis of saliva may be useful tool for the diagnosis and monitoring of infectious and malignant diseases, hereditary disorders autoimmune diseases and assessment of drug levels in various therapeutic approaches [19].

Therefore, this study was designed to compare the levels of iron, ferritin, ALT and, AST in saliva and serum of thalassemia. The levels of the analytes used for monitoring iron status in thalasemia patients were compared to measure statistical difference in serum and saliva.

Materials and Methods

Whole blood and saliva samples of previously diagnosed thalassemia patients were collected (with consent) from Thalassemia Center of Sir Ganga Ram Hospital and Sundas Foundation Lahore, after the approval of Advanced Scientific and Research Board (ASRB) and Ethical Review Committee of University of Health Sciences. Research was conducted in Department of Chemical Pathology UHS Lahore. Samples were collected by convenient sampling technique.

Inclusion Criteria: Previously diagnosed cases of β-thalassemia major patients were included in study.

Exclusion Criteria: Patients with the history of periodontal disease gums and oral bleeding, malignancy and other haemoglobinopathies other

than β-thalassemia were excluded from the study population.

All the personal protective equipments were used during sample collections by following aseptic technique. 3 ml of venous blood sample was collected in a plain tube and centrifuged at 3,000 RPM for 5 minutes to separate the serum. Serum was transferred to sterile Eppendorf tube (3ml) and stored at -4 ˚C until analysis.

In sterile containers, unstimulated saliva samples were collected by spit method. Saliva samples were collected during morning (between 9 and 11 am) to minimize the effects of diurnal variation in salivary composition. The saliva samples were then centrifuged at 3000 rpm for 15 minutes to separate supernatants and stored in three aliquots at -4 ˚C until analyzed.

The levels of ALT and AST in serum and saliva samples in all subjects were measured on chemistry analyzer Erba Chem-7 (Erba Diagnostics USA) by using Randox reagent kit (RANDOX Diagnostics USA).

Alanine aminotransferase (ALT) is an aminotransferase whose enzymatic activity similar to AST. The typical assay procedure for ALT consists of a coupled enzymatic reaction using LDH as the indicator enzyme, which catalyzes the reduction of pyruvate to lactate with the simultaneous oxidation of NADH. The change in absorbance at 340 nm measured continuously, is directly proportional to ALT activity. Assay methods for AST are generally based on the principle, which incorporates a coupled enzymatic reaction. AST catalyses the transfer of an amino group from aspartate to 2-oxoglutarate forming glutamate and oxaloacetate. The rate of decrease in concentration of NADH is proportional to the concentration of AST in the sample using malate dehydrogenase as the indicator reaction and monitors the change in absorbance at 340 nm continuously as NADH is oxidized to NAD+.

Quality control analysis was done by running quality control material on machine and observed the values on L-J chart. Two quality control materials were run before analysis one is healthy control as level1 and other abnormal control as level 2 both control values were within range as given by the manufacturer for quality control material.

Quality Control	Level 1	Level 2	Within Range
ALT	21.5 IU/L	85 IU/L	Yes
AST	16.3 IU/L	97 IU/L	Yes

Iron levels were measured in serum and saliva by ferrozine chromogenic method by using colorimetric iron assay kit (Catalog #No: CFE-005) and absorbance were taken on absorbance micro plate reader (Bio-RAD analyzer) with 560 nm capability.

Below mentioned protocol was used in determination of serum and salivary iron levels.

40 ul of distilled water/Standard and samples were added in their respective wells. Then 200 ul of reagent 1 was added in each well and incubated at room temperature for 5 minutes. After incubation first OD (Absorbance) was taken at 560 nm using BioRad plate reader. 8 ul reagents 2 were added and second OD (Absorbance) at 560 nm was taken after 5 min incubating the plate at room temperature.

Table 1: Measuring density and interpretation of Iron assay

Quality Control	Level 1	Level 2	Within Range
ALT	21.5 IU/L	85 IU/L	Yes
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Wells	OD1	OD2	ΔOD (OD2-OD1)	Iron (µg/dL)
DW (Blank)	0.010	0.020	0.01	-
Standard	0.03	0.101	0.07	-
Sample	0.196	0.144	0.052	148

Description: Measuring range of the employed iron kit was 5-1000 µg/dl.

Serum and saliva ferritin was measured by enzyme-linked immune-sorbent assay (ELISA) method. The procedure was performed on Human FE (Ferritin)

ELISA Kit by using semi-automated ELISA instrument of Bio-Rad Diagnostics. Catalogue number of Human Ferritin was EH0382.

Statistical Analysis

The data was analyzed using SPSS 20.0 (statistical package for social sciences). Normality of the data was checked with Shapiro-Wilk test. Median was given for quantitative variables like Iron, Ferritin, ALT, AST, and Age. Comparison of the results for the two related groups was analyzed by using Paired-Tt test however as the data was not normally distributed so non parametric test Wilcoxon's sign rank test for two related groups was applied instead of Paired-T test. *P-value* of ≤ 0.05 was considered as statistically significant**.**

Results

A total of 40 transfusion dependent diagnosed cases of beta thalassemia major patients aged between 9-22 (12 ±5.15) years were included in the study. From each patient two samples were collected one is the serum sample and other is saliva sample and they were grouped into two groups. Group 1 was the serum group and group 2 was saliva group.

Table 2: Age and Gender Distribution in Study Population

Variables		Patients
Age		12 ±5.15
Gender	Male	21 (52%)
	Female	19 (47%)

Comparison of the results was analyzed by using Paired-t test however as the data was not normally distributed non parametric test Wilcoxon's sign rank test for two related groups was applied instead of Paired-t test and we compared the median values instead of mean values.

Iron, ferritin and aminotransferases were measured in serum and saliva. Median serum iron values were 94.600 µg/dL as the normal values of iron in serum are 55–160 µg/dL. Median saliva iron values were 15.9050 µg/dL as the normal value of saliva iron are 74.20 µg/dL [16]. There is statistically significant difference between serum and saliva iron values as shown by *p*-value < 0.05. Median serum ferritin values were 1074.630 ng/ml as normal ferritin serum values are (20 to 500 ng/ml) [23].Median saliva ferritin values were 10.6120 ng/ml as normal ferritin saliva values are (94.7 ng/ml) [20]. There is statistically significant difference between serum and saliva ferritin values as shown by *p*-value < 0.05. ALT values in serum samples were significantly different from saliva sample values.

The median serum ALT values were 36.50 IU/L as normal serum ALT values are (29 to 33 IU/L) [23]. Median saliva ALT values were 14.000 IU/L as normal saliva ALT values are (3.59 ± 0.65 IU/L) [17]. There is statistically significant difference between serum and saliva ALT values as shown by *p*-value < 0.05. Median values of serum AST were 53.0000 IU/L as normal serum ALT values are 8 to 48 U/L [24]. Median saliva values were 25.00 IU/L as normal saliva AST values are (8. 34 ± 2.13 IU/L) [17]. A statistically significant difference was found between the serum and saliva AST values (*p*-value <0.05).

Table 3: Serum and Saliva values of Iron, Ferritin and aminotransferases in beta Thalassemia Patients. *p-value < 0.05, is significant for all variables. *Median (IQR) given for each variable

Variables	Groups	Median (Q1 -Q3)**	Maximum	Minimum	*p*-value
Ferritin ng/ml	Serum	1074.6300 876.2-1103.8	1130.25	28.36	< 0.05
	Saliva	10.6120 8.29-11.07	12.13	1.75	
Iron µg/dL	Serum	94.6000 66.7-114.7	451.00	14.28	< 0.05
	Saliva	15.9050 11.5 – 24.8	102.85	2.54	
ALT (IU/L)	Serum	36.5000 28 -58	270.00	19.00	< 0.05
	Saliva	14.00 9.2 – 21	122.00	5.00	
AST (IU/L)	Serum	53.0000 37.3 – 86	241.00	23.00	< 0.05
	Saliva	25.0000 15.3 – 30	70.00	7.00	

Table 4: Separate Serum values of Iron, Ferritin and aminotransferases.

Variables	Median (Q1 -Q3)**
Ferritin ng/ml	1074.6300(876.2-1103.8)
Iron µg/dL	94.6000(66.7-114.7)
ALT (IU/L)	36.5000(28 -58)
AST(IU/L)	53.0000(37.3 – 86)

This table describes quartile range of Iron, Ferritin and aminotransferases in serum of *Thalassemia* patients.

Discussion

Thalassemia's are among the common hereditary disorders in the Asian population. Excessive transfusion has been used over the decade that has improved the life span of thalassemic patient but unfortunately iron overload is a major complication that is unpreventable in thalassemic major patients as a consequence of hyper-transfusions [21]. An adequate management to control iron overload is inevitable which can be manifested by frequent evaluation of iron stores in body. Therefore, there is a need to monitor body iron intermittently with quantitative, non-invasive procedures that are accurate and readily available [22]. In addition to blood other body fluids are employed for disease diagnosis however, saliva offers some extra ordinary advantages. Disease diagnosis with the help of saliva is preferably convenient for patients of older age and children due to its easy applicability and non-invasive approach, therefore the study was designed to compare the analytes in serum and saliva of beta thalassemia major patients [19].

The present study was in agreement with the study conducted by Canatan and Akdeniz who inducted 71 patients with transfusion dependent β-thalassemia major between 6-23 (11.6±4.45) years of age. The concentration of iron can be evaluated by various methods in iron overload condition. In the present study normal level of serum iron was observed in beta-thalassemia major patient's which are in agreement with the study carried out by Karim, *et al.*, in 2016. They reported normal iron levels in beta thalassemia major patient. A study on serum ferritin done in Turkey reported increased level of ferritin in serum the result of this study was in agreement with the present study which has increased serum ferritin level as compared to saliva level [16]. Salivary ferritin level were decreased than normal in the present study, these results do not match with another study who analyzed salivary ferritin levels in children with iron deficiency anemia and they found raised ferritin levels [18]. The level of serum ferritin in this study were raised above normal as this study matches with a study conducted in Iraq and found increased level of ferritin in serum of beta thalassemia major patients [25]. In the present study, we measured the levels of iron and ferritin in serum and saliva of beta thalassemia major patient our study agrees with the study done by Canatan and Akdeniz 2012. The researcher of this study measured iron and ferritin levels in saliva and serum of patients with thalassemia and iron deficiency anemia.

As we measured serum and salivary iron and ferritin levels in beta thalassemia major patient and the result of our study shows that there is statistically significant difference between serum and saliva iron and ferritin levels in thalassemia patients so this proves that we cannot use salivary levels of iron and ferritin to monitor iron overload in these patient. The aminotransferases were performed in this study to detect signs of liver damage. In this study both aminotransferases (ALT, AST) levels were measured in serum and saliva of beta thalassemia major patients that are in agreement with another study carried out at the Father Muller Medical College and Hospital, Mangalore. The researcher of the study found increased level of aminotransferases in serum and saliva to detect liver damage in alcoholic's patients [17]. The present study reported increased level of aminotransferases in serum of thalassemic patients this study is similar to study conducted in Mosul, Iraq who recruited beta thalassemia major patients for assessment of liver function and reported increased level of aminotransferases in serum of these patients [25]. These result and statistical difference among values we concluded that although some of the values are raised in saliva however that raise is not enough to use salivary values in management and detection of iron overload in beta thalassemia major patients. A very limited data is available on our study we could not be able to compare our result with other studies of Pakistan due to lack of such researches in our region. We found levels of aminotransferases, iron and ferritin in saliva but not to the level of serum there is a statistically significant difference between serum and saliva values because we have no salivary ranges that are a big limitation of our study.

Conclusion

This study concludes that there is statistically significant difference between serum and saliva values of iron, ferritin and aminotransferases in beta thalassemia major condition. Hence saliva cannot be used as substitute marker of iron overload in beta thalassemia major patients.

Study Outcomes

Saliva cannot be used as an alternate to blood to detect liver damage and iron overload in β-thalassemia major patients.

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