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Iron Overload and Serum and Saliva Ferritin Levels in Individuals with Beta Thalassemia Needing Several Blood Transfusions

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Abstract

Iron overload in individuals with beta thalassemia major is mostly caused by blood transfusion treatment. Since the human body lacks a method for excreting extra iron, iron overload is unavoidable in individuals with thalassemia major who require regular blood transfusions. Because exfoliative cytology is a rapid, easy, painless, and bloodless treatment, it is an attractive assessment. The main intracellular iron-storage protein, ferritin, is released in minute amounts into the body's plasma and helps to maintain iron in a soluble, non-toxic state. In the absence of inflammation, the amount of the body's total iron reserves is positively correlated with the concentration of this plasma (or serum) ferritin. When compared to blood, saliva offers major biochemical and logistical benefits, making it one of the most essential bodily fluids for diagnostic purposes. The diseases associated with iron overload are attributed to the significant increase in ferritin levels in saliva. The objectives of the study were as follows: 1. To measure the levels of iron overload in patients with beta-thalassemia major using oral exfoliative cytology with the special Perl's Prussian blue stain. 2. ELISA was used to measure the ferritin level (iron overload) in the serum and saliva of individuals with beta thalassemia major. 3. To compare the amounts of ferritin in their serum and saliva with the Prussian blue staining positivity of each individual. Smears were extracted from the buccal mucosa of thirty healthy people in the same age range (6-26 years) and sixty β-thalassemia major patients who had received at least ten transfusions. Prussian blue stain kit from Perl was used to stain smears. To estimate ferritin levels, blood and saliva samples were simultaneously obtained from the control and study group. Positivity of Prussian blue was evaluated using predetermined grading standards. 48 out of 60 thalassemic patients (80%) had Perl's positivity, which was positively correlated with ferritin levels in the saliva and serum. Patients with β-thalassemia major can have their iron overload evaluated using coloring peeled cells from the oral mucosa.

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Introduction

Beta thalassemias, sometimes referred to as β thalassemias, are a group of genetic blood disorders. These types of thalassemia vary in severity, ranging from severe anemia to cases that show no clinical symptoms, and this condition results from a deficiency or absence of the production of the beta chain of hemoglobin [1]. Children who have this condition may experience problems that might affect the spleen and need regular

Vol 1, No 1 (2012) DOI 10.5195/d3000/2025.920

blood transfusions for the rest of their lives. Certain children may benefit from bone marrow transplants [2]. Two factors that contribute to or exacerbate iron overload in patients include frequent blood transfusions. Organ damage must be avoided with the use of iron chelation therapy. Treatments for thalassemia major are becoming more advanced, allowing patients to live longer with appropriate care [3,4]. Maintaining a hemoglobin level above 9–10.5 g/dl is the recommended course of therapy for thalassemia major. There are several problems associated with long-term transfusions [5]. The body stores iron mostly in ferritin [6]. A positive acute phase response protein, ferritin no longer reflects the amount of the iron reserve when concentrations rise during inflammation [7]. With considerable biochemical and logistical benefits over blood, saliva is one of the most crucial bodily fluids for diagnostic purposes. According to Bigler et al. (2009) [8], saliva collection is a common procedure that is safe, noninvasive, and somewhat easy for patients to undergo without experiencing any pain.

Ferritin levels in saliva are known [9,10], and in iron overload conditions, salivary ferritin levels rise noticeably. However, the ratio of salivary to serum ferritin does not change, indicating that salivary and serum ferritin grow proportionately while maintaining a stable value [11,12].

When a patient with persistent anemia requires frequent blood transfusions, transfusional iron excess occurs. While the body excretes only around 1 mg of iron per day [13], transfusions include over 250 mg of iron [14]. According to Greer et al. (2013) [3], afflicted patients' liver, heart, endocrine glands, and lungs all have deposits of hemosiderin. Çaliskan et al. (2011) [15] and Rajput et al. (2010) [16] have reported the discovery of iron deposits in the gingivae.

The following tests are used to monitor the patient's iron overload status and the effectiveness of iron chelation therapy: serum ferritin level, serum iron level, total iron-binding capacity, non-transferrin-bound iron level in serum, liver biopsy to assess parenchymal and reticuloendothelial stores, in addition to computed tomography of the liver or magnetic resonance imaging, and cardiac imaging using magnetic resonance imaging (T2 * or Ferriscan technique) [17].

The process of exfoliative cytology is rapid, easy, painless, bloodless, and noninvasive [18]. It is predicated on the microscopic examination of epithelial cells following the fixation and staining process. There are two approaches used: the direct technique, which involves rubbing mucosal surface cells, and the indirect way, which involves aspirating individuals with self-exfoliated cells [19]. Using an affordable method called exfoliative cytology [20], this study aims to quantify the iron excess in such individuals.

Materials and Methods

In the Hereditary Blood Disease Center, Gynecology, Obstetrics, and Pediatrics Department of Ibin Albaladi Hospital in Baghdad, a cross-sectional research was carried out. The research on prominent cases of β-thalassemia who received ten or more blood transfusions was carried out from January to April 2024. The Baghdad University, College of Dentistry's institutional ethics committee gave its approval. The hours of the sample sessions were restricted to 9:00 a.m. to 11:00 a.m. Data from patient files were used to determine the diagnosis and total number of transfusions.

The Sample

Ninety volunteers of both genders, ranging in age from 6 to 26 years, were included in this study and were categorized into two main groups:
60 beta thalassemia major patients were divided into the following study groups: a. Fifteen patients (15) who had received repeated transfusions but were not getting iron chelation therapy (treatment-free). b. Forty-five patients (45) receiving iron chelation therapy were divided into subgroups based on how long their treatments would last (5–10), (11–15), and (16–20) years.
2. 30 clinically and haematologically healthy people made up the control group.

Inclusion Criteria

1. Patients between the ages of 26 and 26 who regularly get blood transfusions with a transfusion count of at least 10.
2. The patient's inclusion was confirmed by hemoglobin electrophoresis.
3. Control group: Individuals chosen based on a clinical examination, history, and blood tests that were within normal limits.

Exclusion Criteria

Individual who has not yet received a blood transfusion and who has a history of any other serious illnesses. Hepatitis, megaloblastic anemia, iron deficiency anemia, cancer, and verified acute and chronic liver injury should all be absent in control persons.

Sample Collection

At the same time, swabs, saliva samples, and blood samples were collected. Patients were instructed to rinse their mouths with distilled water to remove any residues., both in the research group and the control group. Patients' buccal mucosa was cleansed with gauze, and cells were then extracted using an interdental brush that was used repeatedly in a straight line and with very little pressure. According to Thomas et al. (2009) [21] and Farhan and Yas (2018) [22], scrapings were spread out across a vast area on a clean, dry, frosted glass slide to prevent cell clumping. Following the fixation of the slides in a coplin jar with 70% ethanol alcohol, the slides were sent to a lab for iron staining using an abcam 150674 kit.

To determine whether the blue iron granules are present in the cytoplasm, the sample was first examined using a 10x magnification lens and then with a 40x magnification lens. For grading purposes, ten high power fields (objective lens x40) that met the following requirements were selected:
1. Cells that are widely apart and barely overlap.
2. In each high-power field (x40), a minimum of 20 epithelial cells and a maximum of 30 cells.
3. Whether blue intracytoplasmic granules are present or absent [20].
A camera that was fixed to the microscope was used to take the pictures. Achromatic objective of 40X was used to take all the cell photos. The computer was used to save the captured images, and Image J software version 1.52 a was used for analysis. The average results were calculated from ten high-

Vol 1, No 1 (2012) DOI 10.5195/d3000/2025.920

strength areas (40X), and then the appropriate score was determined based on that.

- Grade 0 - No particles
- Grade I - Less than 5 particles in the high field of vision
- Grade II - 5-10 particles in the high field of vision
- Grade III - 10-20 particles in the high field of vision
- Grade IV - Visible clumps in less than 3 high fields of vision
- Grade V - Coarse particles or visible clumps in 3 or more high fields of vision [20].

Bone marrow used as positive control tissue.

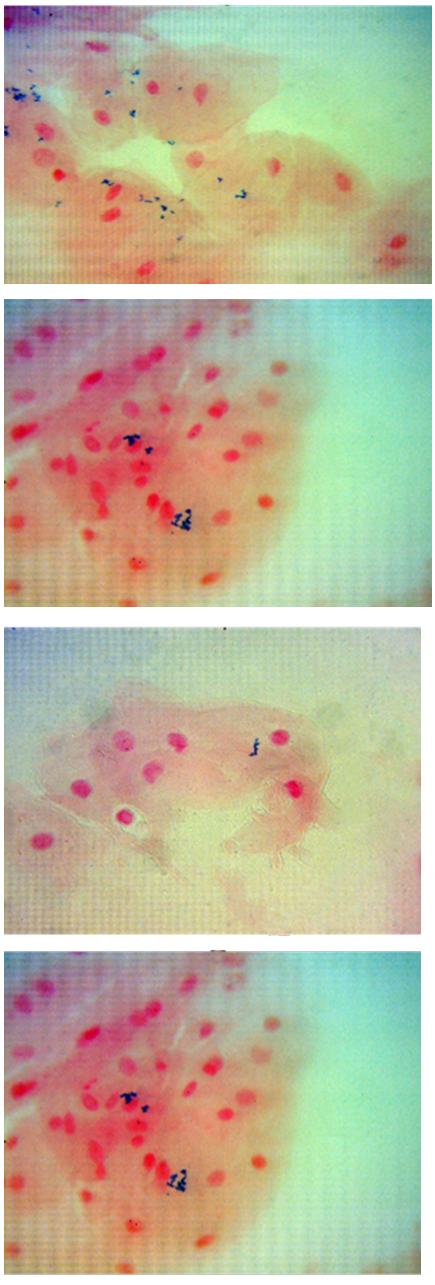
The study and control groups had their venous blood drawn at the same time as exfoliative cytology. The sample was centrifuged for 20 minutes at 4000 rpm to produce a clear supernatant. The hospital next used VIDAS® Ferritin, an automated quantitative test that uses the Enzyme Linked Fluorescent Assay technology with a ferritin kit (bioMérieux) to determine the level of ferritin in human serum or plasma.

Saliva was taken from each participant while they were at rest and not aroused. One hour before to the sample collection, the participants were told not to eat or drink anything and to rinse their mouths with water to get rid of any debris. Under supervision, the participants were told to sit comfortably with their heads slightly leaned forward, allow saliva to collect on the floor of their mouths, and then expel it through expectoration. During the five-minute saliva collection time, the samples were coded, and the patient's identity was recorded. The samples were processed and stored in a sterile plastic specimen container [23].

Saliva samples were collected, then centrifuged for 20 minutes at 4000 rpm, with the clear supernatant removed and put in Eppendorf tubes. The samples were then deep frozen at -20°C until the required number of samples were obtained. The E-EL-H0168 Human FE (Ferritin) ELISA kit/Elabscience was then used to analyze saliva samples in the laboratory.

Statistical Analysis

Data analysis was conducted using the Statistical Package for the Social Sciences (SPSS) version 22.0. The chi-square test was applied to compare the control group and the study group, and Analysis of Variance (ANOVA), Spearman's correlation coefficient, and the F-test were calculated to relate the positivity of the oral swab to serum and saliva ferritin levels.



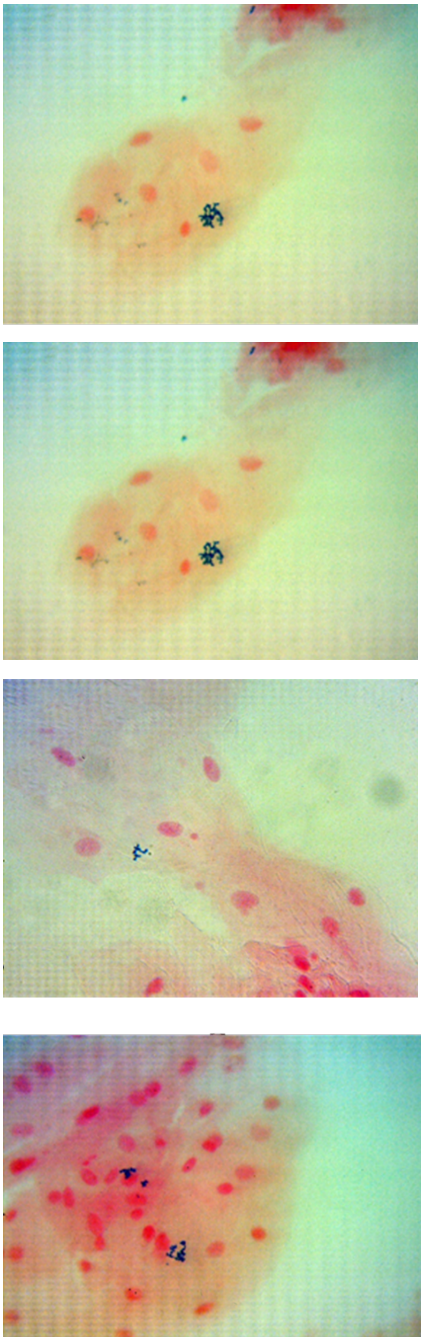


Figure 1. Perl's Prussian blue staining of squamous epithelial cells from buccal mucosa showing blue intracytoplasmic iron granules, counter stain used is nuclear fast red stain.

Results

Among the 60 patients in the research group, 48 patients (80%) showed a positive result for the blue Prussian dye test in the exfoliated oral mucosal cells. Meanwhile, all thirty participants in the control group (100%) showed a negative reaction to the blue

Vol 1, No 1 (2012) DOI 10.5195/d3000/2025.920

Prussian dye. The results of the Prussian dye test were compared between the research and control groups using the chi-square test, and as shown in Table 1, there was a statistically significant difference between the two groups at a p-value of >0.001.

Serum Ferritin (ng/ml) Marker

In reference to "Serum Ferritin" ng/ml across the various groups, the non-treatment group has the highest mean value (8194.3±3549.7) ng/ml with a large variation when compared to the other treated study groups, which have mean levels of (5274.4±2940.9) ng/ml, (5062.7±1926.6) ng/ml, and (4567.3±2102.9) ng/ml for the 5–10, 11–15, and 16–20 years accordingly (Table 2).

Saliva Ferritin

In relation to "Saliva Ferritin" ng/ml, no treatment group recorded the highest mean value (25.347±4.273) ng/ml. Then, treated groups 5-10 years, 11-15 years, and 16-20 years period followed, with mean values (11.319±3.395) ng/ml, (5.021±2.025) ng/ml, and (2.061±1.167) ng/ml, respectively, and lastly, the control group (0.146±0.058) ng/ml. This is shown in Table 3.

Influence between Saliva and Serum Ferritin

A correlation between the examined markers was found (R=0.94254; P<0.001).

Comparison of Pearl's Stain Grades in Exfoliative Cytology and Serum and Saliva Ferritin Levels in the Study Groups

The current study's results, when compared using ANOVA, the F test, and the spearman correlation coefficient, revealed a non-significant association between "Saliva ferritin" and the grades of buccal smears, but a significant correlation (P<0.05) between "Serum ferritin" and the exfoliative cytology grades.

Out of 60 patients, a maximum of 22 (36.6%) had a mean serum ferritin level of 5102.9±2357.5 ng/ml, indicating Grade IV positive. Thirteen patients (21.6%) had a mean serum ferritin level of 5953.7±3254.1) ng/ml, indicating Grade III positivity. Subsequently, 12 patients (20%) had a mean serum ferritin level of 7791.0±3179.3 ng/ml, falling into grade (0). Eight patients (13.3%), on the other hand, had a mean serum ferritin level of 4334.9±2005.3 ng/ml, belonging to grade II. Subsequently, 3 patients (5%) had a mean blood ferritin level of 8307.2±3428.9 ng/ml (grade V). Table 4 illustrates that a mere 2.3% of individuals exhibited grade I, with a mean blood ferritin level of 1862.7%±4688.3% ng/ml.

Discussion

Cells were collected from the oral mucosa of 48 out of 60 patients suffering from severe thalassemia (80%) in this investigation showed positive results for Perls' Prussian blue response. It was also noted that Perls' Prussian blue positive was absent from all the control participants. Like the findings of Gururaj and Sundharam et al. (2003) [24], who reported 100% Perls' staining positivity in the 10 patients they studied, their results also demonstrated Prussian blue staining positivity in Perls.

According to Nandprasad et al. (2009) [25], 65 out of 100 patients had 65% Perls' positivity; Bhat et al. (2013) [26] observed 71.7% positivity (43 out of 60 patients); Chittamsetty et al. (2013) [27] reported 72.5% (29 out of 40 β-thalassemia major patients); and Gupta et al. (2014) [28] observed 61.6% Perls' positivity (37 out of 60 cases). Findings from Leekha et al. (2016) [29] revealed that 35 (87.5%) of 40 patients had favorable results. Furthermore, 98% of Perls' positive (49 out of 50 instances) was recorded by Gajaria et al. (2017) [20].

The observed positive findings variance can be explained by the variations in the mean blood ferritin levels of the patients involved in these investigations, as well as the overall sample size differences among studies [20]. Additionally, a comparison of the study group's Perls' Prussian blue staining with that of the control group demonstrated that the study group's positivity was not coincidental. As a result, there is a strong correlation between the positive iron staining in oral smears of patients with beta-thalassemia major who receive frequent blood transfusions and suffer from hemosiderosis. This indicates that iron accumulates in the squamous epithelial cells of the oral mucosa in cases of hemosiderosis., and that iron overload may be measured by scraping the cells. The controls did not have iron excess and were in good health.

The lack of positive instances in the research group may have resulted from improper staining methods or early shedding of the impacted squamous epithelial cells, even though the patients had elevated serum ferritin levels. The creation of iron storage pools is one of the processes that determines the excess quantities of iron that collect in different tissues. Perls' Prussian blue staining positivity is always impacted by the variation in ferritin amounts generated in exfoliated buccal mucosal cells [20]. According to Delaby et al. (2005) [30], iron initially builds up in reticuloendothelial macrophages and then spreads to parenchymal cells when hemosiderosis happens.

As compared to control participants, the study groups' mean blood ferritin levels were considerably higher, with the no treatment group having the highest mean value. These findings are consistent with Canatan and Akdeniz (2012) [12] and Hassan et al. (2013) [31].

In agreement with Canatan and Akdeniz (2012) [12], the study groups' mean saliva ferritin levels were significantly higher than those of the control subjects. The controlled group also showed highly significant differences, with a P-value of less than 0.001, when compared to the study groups. According to Jaganthan et al. (2012) [10], salivary ferritin levels significantly increase in iron overload illnesses.

Because saliva contains enzymes that are dependent on iron, the biological system maintains larger amounts of salivary ferritin, which helps to preserve iron through saliva [32]. There is not enough information in the literature to pinpoint the precise pathophysiology of salivary ferritin elevation. Although ferritin [12] and salivary components [33] may be similar in both serum and saliva, the synthesis and secretion of parotid gland acinar cells may affect salivary component levels, making them inconsistent with serum [34].

Vol 1, No 1 (2012) DOI 10.5195/d3000/2025.920

The current investigation found a significant association (p>0.05) between the mean serum ferritin levels in the treated and non-treated groups and the grades of buccal smear positive. This finding suggested that the mean serum ferritin levels and buccal smear grades were associated, and the results were found to be comparable to those of Gajaria et al. (2017) [20]. The grade of buccal smear positive was shown to have a strong correlation with the patients' blood ferritin levels; as a result, patients receiving regular transfusions can utilize this straightforward, non-invasive diagnostic to track iron excess.

Many factors, such as the number of blood transfusions the patient has received, the age at which iron chelation therapy began, whether the treatment is taken regularly, social and economic status, nutritional deficiencies, and other comorbidities, affect the increase of iron in this group of patients [20]. Regarding the "Saliva ferritin" marker, there is no discernible relationship with the grades of buccal smears. We are not aware of any research that relates salivary ferritin levels to Perls' Prussian blue staining grades of oral exfoliative cytology.

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Vol 1, No 1 (2012) DOI 10.5195/d3000/2025.920

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Table 1. Comparison of the reaction of Prussian blue of pearls between the study group and the control group using the contingency coefficient.

Groups	Exfoliative cytology staining No&%				Total No.&%		C.S. (*) P-value
	Negative		Positive				
Control	30	100%	0	0.0%	30	100%	C.C. = 0.625
Patients	12	20%	48	80 %	60	100%	P=0.000
Total	42	46.6%	48	53.3%	90	100%	

(*) Significant at P<0.001; Testing based on chi-square.

Table 2. Summary statistics of serum ferritin (ng/ml) readings distributed in different groups.

Groups	No.	Mean	Standard Deviation	Standard Error	95% C.I. for Mean		Minimum	Maximum
					Lower Boundary	Upper Boundary		
Control	30	71.653	47.2	8.6	54.0	89.3	17.6	180.8
No treatment	15	8194.3	3549.7	916.5	6228.6	10160.1	2492.0	12000.0
5-10 years	15	5274.4	2940.9	759.3	3645.8	6903.1	1531.5	11929.4
11-15 years	15	5062.7	1926.6	497.5	3995.8	6129.6	1969.1	8059.8
16-20 years	15	4567.3	2102.9	543.0	3402.8	5731.8	1515.1	7688.6

Table 3. Summary statistics of saliva ferritin (ng/ml) readings distributed in different groups.

Groups	No.	Mean	Standard Deviation	Standard Error	95% C.I. for Mean		Minimum	Maximum
					Lower Boundary	Upper Boundary		
Control	30	0.146	0.058	0.011	0.125	0.168	0.100	0.300
No treatment	15	25.347	4.273	1.103	22.980	27.713	15.050	30.12
(5 _ 10) yrs.	15	11.319	3.395	0.877	9.439	13.199	6.530	17.05
(11 _ 15) yrs.	15	5.021	2.025	0.523	3.899	6.142	1.310	9.500
(16 _ 20) yrs.	15	2.061	1.167	0.301	1.415	2.708	1.100	5.200

Table 4. Comparison of exfoliative cytology grades with average serum and salivary ferritin levels in study groups.

Marker	Grade	No. of Patients	Mean	Standard Deviation	Standard Error	Minimum	Maximum	P-value
Serum Ferritin ng/ml	0	12	7791	3179	917	2501	12000	F=3.333
	I	2	1862	468	331	1532	2194	P=0.011

Vol 1, No 1 (2012) DOI 10.5195/d3000/2025.920

	II	8	4334	2005	709	1984	7374	
	III	13	5953	3254	902	2501	12000	
	IV	22	5102	2357	502	1515	11725	
	V	3	8307	3428	1979	5224	12000	
Saliva Ferritin ng/ml	0	12	10.7	10.4	3.0	1.2	30.1	F=1.625
	I	2	9.5	8.3	5.9	3.6	15.4	P=0.169
	II	8	7.9	4.1	1.5	1.1	15.3	
	III	13	10.7	11.1	3.1	1.1	29.7	
	IV	22	10.5	9.2	2.0	1.1	28.9	
	V	3	25.2	2.0	1.2	22.9	26.5	

Testing based on ANOVA, F test and spearman correlation coefficient.