DOI Number: 10.37506/v14/i1/2020/ijfmt/192958

Iron Overload Estimation by Oral Exfoliative Cytology in Beta Thalassemia Major Patients Undergoing Repeated Blood Transfusion

Anaam Mahdi Hadi[1] Layla Sabri Yas[1]

[1]Department of Oral Diagnosis, College of Dentistry, University of Baghdad/Iraq

Abstract

β-Thalassemia major patients require regular blood transfusion therapy lifelong this lead to iron overload in the body tissues, which is a major cause of morbidity and mortality in these patients. Exfoliative cytology, a non-invasive and inexpensive technique based on microscopic evaluation of epithelial cells after a procedure of their fixation and staining. The aims and objectives of this study were: 1.To estimate iron overload by oral exfoliative cytology using Perl's Prussian blue stain in β-thalassemia major patients. 2. To correlate perl's Prussian blue staining positivity with their serum ferritin levels. Smears were obtained from buccal mucosa of 60 β-thalassemia major patients (who had taken ≥10 transfusions) and 30 healthy subjects of the same age group (5-26) year. Smears were stained with Perl's Prussian blue stain kit. Blood samples were taken from the study and control group at the same time for estimation of serum ferritin levels. Grading criteria were defined for assessing the Prussian blue positivity. Perl's positivity was observed in 48 out of 60 of thalassemic patients (80%), with a positive correlation to serum ferritin levels. Perl's Prussian blue staining of exfoliated cells from buccal mucosa can be used to assess iron overload in β-thalassemia major patients, as a screening tool.

Key words: *β-thalassemia major, iron overload, exfoliative cytology, perl's Prussian blue*

Introduction

Beta-thalassemia syndromes are a group of hereditary blood disorders characterized by reduced or absent beta globin chain synthesis [(1)]. β-Thalassemia major also known as Cooley's anaemia. The recommended treatment for thalassaemia major involves lifelong regular blood transfusions, usually given every two to five weeks, to maintain the hemoglobin level above 9–10.5 g/dl. Long term transfusion has multiple complications [(2)]. Iron stores in the body exist primarily in the form of ferritin [(3)]. Ferritin is a positive acute phase response protein whereby concentrations increase during inflammation and thereby no longer reflect the size of the iron store [(4)]. Transfusional iron overload develops in patients with chronic anaemia who need to have regular blood transfusions. About 250 mg of iron is the unit of transfused blood, while only about 1 mg of iron that the body excreted per day [(3) (5)]. Hemosiderin deposits in the liver, heart, endocrine glands and lungs of the affected patients [(2)]. Iron deposits have also been found in the gingivae [(6) (7)]. Serum ferritin, Serum iron, iron-binding capacity, Serum non-transferrin bound iron, Bone marrow biopsy (Perl's stain) for iron within reticuloendothelial stores, Liver biopsy (parenchymal and reticuloendothelial (RE) stores), Liver CT scan or MRI and Cardiac MRI (T2 * or Ferriscan technique) are methods used to monitor iron overload status and effect of iron chelation therapy in these patients [(8)].

Exfoliative cytology is painless, bloodless, noninvasive, quick and simple procedure [(9)]. it based on microscopic evaluation of epithelial cells after a procedure of their fixation and staining. There are 2 methods in use: the indirect cell collecting method, such as aspiration subjects with Self exfoliated cells, and the direct method, rub cells of mucosal surface [(10)]. This study is an attempt to measure iron overload in those patients by means of exfoliative cytology, an inexpensive technique [(11)].

Aims and Objectives

1. Assessment of iron over load in beta thalassemia major patients by oral exfoliative cytology using perl's

Prussian blue stain.

2. Correlation of perl's Prussian blue staining with serum ferritin levels.

Materials and Method

This cross sectional study was conducted in Ibin Albaladi hospital for gynyaecology, obstetric and pediatrics -thalassemia center, in Baghdad during the period from January to April 2019. Sampling sessions were limited to the hours between 9:00 and 11:00 AM, data about diagnosis and number of transfusions were taken from patients' files.

The present study comprised of 90 subjects, with an age range of (5-26) years of both gender, broadly classified into two groups:

1. study group comprised of 60 beta thalassemia major patients

2. Control group: comprised of 30 clinically and hematologically healthy individuals

Inclusion criteria:

1. Patients aged (5-26) year, receiving regular blood transfusion ≥10 transfusion.

2. Patient was included based on confirmation by hemoglobin electrophoresis.

3. Control group: clinically and hematologically healthy individuals, in the age range (5-26) years. Subjects selected on the basis of history, clinical examination and blood investigations within normal range.

Exclusion criteria: subject with history of any other major illness and newly diagnosed cases yet to receive blood transfusion. Control subjects should have no confirmed acute and chronic liver damage, malignancy, megaloblastic anemia, iron deficiency anemia, and hepatitis.

Sample collection.

Patients from the study group and the control group were asked to rinse their mouth with distilled water to remove any debris. Buccal mucosa of the patients was cleaned by guaze and then cells from buccal mucosa were collected by using interdental brush. Scraping were placed on the middle of clean and dry frosted glass slide and spread over a large area to avoid clumping of cells [12] [13]. Slides then placed in coplin jar containing 70% ethanol alcohol for fixation and transferring to laboratory for staining by iron staining kit (abcam 150674).

The smear was first examined at x10 lens followed by examination at x40 lens to study the presence or absence of intra cytoplasmic blue-colored iron granules.

Ten high power fields (objective lens x40) fulfilling the following criteria were chosen for grading purpose:

1. Evenly dispersed cells with minimal overlapping.

2. Minimum 20 epithelial cells and maximum30 cells per high power field(x40).

3. presence/absence of blue intra cytoplasmic granules [11].

The images were captured with a camera attached to the microscope. All the images of the cells were captured with x40 achromatic objective. Captured Images were stored on the computer and analysis was done using the software image J software version 1.52 a. An average of findings in 10 high power fields (x40) were taken and accordingly, appropriate grade assigned.

- Grade 0 - No granules
- Grade I – less than 5 granules/high power field
- Grade II—5–10 granules/high power field
- Grade III—10–20 granules/high power field
- Grade IV—Clumps seen in less than3 high power field
- Grade V—Coarse granules or clumps seen in 3 or more than 3 high power field [11].

Venous blood was collected at the same time of taking exfoliative cytology from study and control groups, the sample were centrifuged at 4000 rpm for 20 min to get clear supernatant. then serum ferritin were estimated in the hospital by VIDAS® Ferritin which is an **automated** quantitative test for the determination of ferritin in human serum or plasma using the Enzyme Linked Fluorescent Assay technique using ferritin kit (bioMérieux) .

Figure1: 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, positive control tissue used is bone marrow.

Statistical analysis

Data analysis was performed using statistical package (SPSS) ver. (22.0), Chi square test was applied to compare the control and study groups, Analysis of variance (ANOVA) was used and F-test calculated to correlate the buccal smear positivity with serum ferritin levels.

Results

In this study 48(80%) patients out of 60 in the study group showed positivity for the Perl's Prussian blue staining in the exfoliated cells of the buccal mucosa. Maximum number of patients, 22 (36.6%) out of 60 had Grade IV positivity, followed by 13 (21.6%) patients having Grade III while 8 (13.3%) patients was Grade II, 3(5%) patients having grade V, 2 (3.3%) patients having grade I and 12 (20%) patients have grade 0 (negative for perl's Prussian blue staining). All 30 subjects in the control group (100%) were negative for Perl's Prussian blue stain. Chi square test was applied to compare the Perl's staining of the control and study groups. It was found to be statistically highly significant at 1% level of significance (p value> 0.001).

Table (1): Comparison of Perl's Prussian blue reaction in β-thalassemic patients and control subjects with a contingency coefficient

Groups	No. & %	Exfoliative cytology		Total	C.S. (*) P-value
		Neg.	Pos.		
Control	No.	30	0	30	C.C. = 0.625 P=0.000 HS
	%	71.4%	0.0%	33.3%	
Patients	No.	12	48	60	
	%	28.6%	100.0%	66.7%	
Total	No.	42	48	90	
	%	100%	100%	100%	

(*) Highly Sig. at P<0.01; Testing Based on a Contingency Coefficient.

In order to correlate the buccal smear positivity with serum ferritin levels, ANOVA was used and F-test calculated. The comparison of buccal smear grades of the study group was made with their respective mean serum ferritin levels. This was again found to be significant at 5% level of significance implying that buccal smear grades correlated with the mean serum ferritin levels, as shown in table(2):

Table (2): Summary Statistics Comparison of buccal smear grades with the respective mean serum ferritin levels of the study group

Marker	Grade	No. of Patients	Mean	Std. E.	Std. Dev.	Min.	Max.	P-value
Serum Ferritin ng/ml	0	12	7791.0	3179.3	917.8	2501	12000	F=3.333 P=0.011 S
	I	2	1862.7	468.3	331.1	1532	2194	
	II	8	4334.9	2005.3	709.0	1984	7374	
	III	13	5953.7	3254.1	902.5	2501	12000	
	IV	22	5102.9	2357.5	502.6	1515	11725	
	V	3	8307.2	3428.9	1979.7	5224	12000	

(*) S: Sig. at P<0.05; Testing Based on ANOVA.

Result has recorded significant relationship at P<0.05 in light of "Serum Ferritin ng/ml"

Discussion

Blood transfusion therapy being the major cause of iron overload in thalassaemia major. When thalassaemia major patients receive regular blood transfusion, iron overload is inevitable because the human body lacks a mechanism to excrete excess iron [10].

In this study, exfoliated cells from the buccal mucosa of 48 of the 60 thalassemic patients (80%) group revealed positivity for Perl's Prussian blue reaction. Further, it was observed that none of the control subjects showed Perls' Prussian blue positivity. These results of Perl's Prussian blue staining positivity were similar to Gururaj and Sundharam *et al* study (2004) who reported 100% Perl's positivity in the 10 patients that they examined [14], Nandprasad *et al* study (2010) who observed 65% Perl's positivity (65 out of 100 patients), Bhat et al study (2013) who reported 71.7% positivity (43 patients positive out of 60), Chittamsetty *et al* study (2013) who observed 72.5% (29 out of 40 b-thalassemia major patients) and Gupta *et al* study (2014) who observed 61.6% Perl's positivity (37 out of 60 cases) [15-18], Leekha *et al* study (2016) who found Values to be positive in 35 (87.5%) out of 40 patients [19], And Gajaria *et al.* study (2017) who observed 98% perl's positivity (49 out of 50 cases). In the present study, result is higher than of Nandprasad et al. study (2010), Bhat *et al*. study (2013), Chittamsetty *et al.* study (2013 and Gupta *et al*. study (2014), but lower than Gururaj and Sundharam *et al.* study (2004) and Gajaria *et al.* study (2017). This variation in positive results can be attributed to the difference in the total sample size of various studies as well as the varying mean serum ferritin levels of the respective patients in the studies [11]. Also comparison between the study and control group Perl's Prussian blue staining implied that positivity in the study group was not by chance. Thus, there was a strong association between the thalassaemia patients who were undergoing repeated blood transfusions and suffering from hemosiderosis with their buccal smear iron stain positivity. This proved that iron does accumulate in squamous epithelial cells of buccal mucosa in hemosiderosis and these cells can be scraped to assess iron overload. The controls were healthy and did not suffer from iron overload explaining their negative results. The negative reaction in study group even in spite of high serum ferritin levels in those patients can be explained by either fault in the technique of staining or already shedding of the affected squamous epithelial cells. The excess amount of iron that gets accumulated in various tissues is dependent upon various factors including the formation of iron storage pool. Moreover, amount of ferritin formed in exfoliated buccal cells, may vary, which may invariably affect the Perl's positivity [11]. When hemosiderosis occurs, iron accumulates in reticuloendothelial macrophages first and only later spills over into parenchymal cells [20]. It was observed that the grade of buccal smear positivity correlated well with the serum ferritin levels of these patients and therefore this simple, non-invasive test can be used to monitor iron overload in patients taking regular transfusions.

Nandprasad *et al* study. And Chittamsetty *et al* study. Had included the patients with 15 or more number of blood transfusions in their study [15-17]. In Gajaria *et al* study (2017), Perl's Prussian blue positivity was observed even with the number of transfusions being (12), in this study positivity observed with (10) transfusions implying that this test can be used as a screening modality also, besides regular monitoring. Most of these patients were on chelation therapy, yet Perl's Prussian blue positivity was detected in their buccal smears further validating the usefulness of this test.

The various factors affecting the iron overload in this subset of patients are number of transfusions, age of initiation of iron chelation therapy, whether taking the chelation therapy regularly, socioeconomic status, nutritional deficiencies and other associated comorbidities.

Studies to compare the Exfoliative cytology Perl's Prussian blue positivity with liver biopsy, T2* MRI of liver/heart, MUGA scan (Multigated acquisition), liver dry weight iron need to be conducted. Also a co-relation of this test with clinical manifestation of iron overload in various organs needs to be done.

Conclusion

Perl's Prussian blue reaction can be utilized as an objective indicator of iron overload in β-thalassemic patients with high levels of iron overload.

Perl's Prussian blue staining of exfoliated cells from buccal mucosa can be used to assess iron overload in β-thalassemia major patients, and as a screening modality to detect iron overload in patients yet to start chelation therapy. With the grading system we can even give a

semi quantitative assessment of the same. Since these grades correlated positively with the respective serum ferritin levels, it can be used to monitor iron overload in those Patients.

Acknowledgements: We are grateful to the staff of thalassemia center, blood bank and hematology laboratory in Ibin Albaladi hospital for gynyaecology, obstetric and pediatrics in Baghdad and would also like to acknowledge the hard work of our senior and staff of oral pathology laboratory in Baghdad university/college of dentistry.

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq

Conflict of Interest: The authors declare that they have no conflict of interest.

Funding: Self-funding

References

1. Ja'afar MN, Dawood F. Al-Aswad. Oro-facial manifestations, microbial study and salivary enzyme analysis in patients with â-Thalassemia Major. *Journal of Baghdad College of dentistry*. (2012); *24*(1):52_56.
2. Greer JP, Arber DA, Glader B, List AF, Means RT, Frixos P, Rodgers GM. Wintrobe's Clinical Hematology, 13th edn. Wolters Kluwer Lippincott Williams and Wilkins Health. 2014.
3. Theil EC. Ferritin protein nanocages—the story. *Nanotechnology perceptions*. 2012; *8*(1): 7.
4. World Health Organization, World Health Organization. Iron deficiency anaemia: Assessment, *Prevention and Control. A guide for programme managers. Geneva: WHO*. 2001; 99.
5. Kzar MY, Hussein AM. Salivary Iron and Ferritin Levels, Orofacial Complications of Patients with Thalassemia Major In Babylon Teaching Hospital For Maternity and Children. *Medical Journal of Babylon*. 2017; *14*(1):135-139.
6. Çaliskan U, Tonguç MÖ, Çiris M, Balta N, Kirzioglu FY, Çaglayan G, Canatan D. The investigation of gingival iron accumulation in thalassemia major patients. *Journal of pediatric hematology/oncology*. 2011;*33*(2):98-102.
7. Rajput DV, Tupkari JV. Early detection of oral cancer: PAP and AgNOR staining in brush biopsies. *Journal of oral and maxillofacial pathology: JOMFP*. (2010);*14*(2):52.
8. Hoffbrand AV, Moss PA. *Hoffbrand's essential haematology* (Vol. 38). John Wiley & Sons. 2015.
9. Sivapathasundharam B, Kalasagar M. Yet another article on exfoliative cytology. *Journal of Oral and Maxillofacial pathology*. 2004;*8*(2):54.
10. Cappellini MD, Cohen A, Porter J, Taher A, Viprakasit V. *Guidelines for the management of transfusion dependent thalassaemia (TDT)* . Nicosia, Cyprus: Thalassaemia International Federation. 2014.
11. Gajaria PK, Maheshwari UM, Borkar DB, Dhar R, Pancholi V. Buccal Mucosa Exfoliative Cell Prussian Blue Stain Co-Relates with Iron Overload in β-Thalassemia Major Patients. *Indian Journal of Hematology and Blood Transfusion*. 2017;*33*(4):559-564.
12. Thomas P, Holland N, Bolognesi C, Kirsch-Volders M, Bonassi S, Zeiger E, Fenech M. Buccal micronucleus cytome assay. *Nature protocols*. 2009;*4*(6):825.
13. Farhan RS, Yas LS. Oral Finding and Cytomorphometric Analysis of Oral Mucosal Cells in Type 2 Diabetic Patients. *International Journal of Medical Research & Health Sciences*. 2018;*7*(6):86-93.
14. Gururaj N, Sundharam S. Demonstration of iron in the exfoliated cells of the oral mucosa. J Oral Maxillofac Pathol. 2003;1:37–39
15. Nandprasad S, Sharada P, Vidya M, Karkera B, Hemanth M, Prakash N. Oral exfoliative cytology in beta thalassemia patients undergoing repeated blood transfusions. Internet J Pathol. 2009;10:7.
16. Bhat Atul A, Parwani RK, Wanjari SP. Demonstration of Iron in exfoliated buccal cells of b thalassemia major Patients. J Cytol. 2013;30(3):169–173.
17. Chittamsetty H, Munishekar Syed, Ahmed Afroz, Palla Churu Suri Sridevi . A Non- invasive Technique which demonstrate iron in the buccal mucosa of sickle cell anaemia and thalessemia patients who undergo repeated blood transfusion. J Clin Diag Res. 2013;7(6):1219–1222.
18. Gupta S, Trichal VK, Malik R, Nigam RK, Choudhary R, Shrivastava A. Perl's prussian blue positivity in exfoliated buccal cells of b thalessemia

major patients and its correlation with serum ferritin. J Evol Med Dental Sci. 2014;3(19):5135–5140.

19. Leekha S, Nayar AK, Bakshi P, Sharma A, Parhar S, Soni S. Estimation of iron overloads using oral exfoliative cytology in beta-thalassemia major patients. *CytoJournal*. 2016;*13*.

20. Delaby C, Pilard N, Gonçalves AS, Beaumont C, Canonne-Hergaux F. Presence of the iron exporter ferroportin at the plasma membrane of macrophages is enhanced by iron loading and down-regulated by hepcidin. *Blood*. 2005;*106*(12):3979-3984.