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Demonstration of Iron in Exfoliated Buccal Mucosal Cells as a test for Iron Overload in Thalassemia Syndrome Patients Undergoing Repeated Blood Transfusion

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Introduction

Thalassemias are common genetic disorders in the Indian subcontinent.[1] Thalassemia major is the severe phenotype which requires lifelong transfusions and bone marrow transplantation is the only curative option available.[2]

Approximately 7% of the world's population is a carrier for hemoglobin disorders with 300,000–500,000 births every year with the severe heterozygous form of disease.[3] Beta thalassemia is the commonest inherited hemoglobin disorder in the Indian subcontinent with an uneven distribution among the different endogenous populations. There are an estimated 200 million carriers of the β-thalassemia gene worldwide, 20 million being in India.[2] Carrier frequency ranges between 3.7 and 10%[4].

Anemia is the most common clinical presentation of thalassemia. Depending upon the type of the disease and varying severity of anemia, thalassemia further classified into transfusion dependent thalassemia (TDT) and non-Transfusion dependant thalassemia (non-TDT). The TDTs require regular blood transfusion to survive. Without adequate transfusion, they would suffer several complications and a short life span.[5]

Patients of above categories, need regular blood transfusion. The recommended treatment for thalassaemia major and some form of Eβ-thalassemia requires lifelong regular blood transfusions, usually administered every two to five weeks. The goal is to maintain the pre-transfusion haemoglobin level above 9-10.5g/dl. This transfusion regimen promotes normal growth, allows normal physical activities, suppresses bone marrow activity in most patients and minimize transfusional iron accumulation.[5]

Iron deposition in parenchymal tissues begins within 1 year of starting the regular transfusions.[6,13] The iron burden in the body can be estimated by means of the serum ferritin, serum iron, and total iron binding capacity levels. The estimation of serum ferritin level is the most commonly employed test to evaluate iron overload in these patients.

Thus Thalassemia patients need regular monitoring of serum ferritin to prevent iron overload and to monitor iron chelation therapy. But the serum ferritin assessment needs regular

venipuncture, skilled technician and good laboratory facility. Again Liver iron correlates closely with the total body iron in transfusional iron overload.[6] Measurement of the iron concentration in a liver biopsy specimen is a reference method for assessing the body iron stores. Other methods for evaluation of hepatic iron include superconducting-quantum-interference-device (SQUID), liver magnetic resonance imaging (MRI) and magnetic spectrometry.[6,7] The discomfort and the potential risk associated with the liver biopsy procedure as well as the availability and cost factor associated with MRI and SQUID have prompted us to search for non-invasive and cheaper method for evaluation of iron stores.

Oral exfoliative cells of thalassemia patients when stained with Perls' Prussian Blue stain technique gives blue coloration of intracytoplasmic granules which indicates iron overload in body. This oral exfoliative cell collection is easy, non-invasive and can be done by any health personnel without necessity of higher skill. The staining method is also easy and cost effective. In this study we observed the correlation of serum ferritin level and presence of intracytoplasmic granules in buccal mucosal cells which indicates iron overload in thalassemia cases undergoing repeated blood transfusions.[8] So in our study, We wanted to see whether exfoliative cytology could be a useful tool in detection and monitoring of iron overload in thalassemia syndrome patients who are undergoing repeated blood transfusion. This simple and cost-effective investigation may help in prevention of iron overload and morbidity & mortality associated with the same in these patients.

Aims and Objectives

1. Evaluation of feasibility of oral exfoliative cytology using Perls' Prussian blue stain to assess iron overload in Thalassemia syndrome patients.
2. To correlate Perls' Prussian blue staining with serum ferritin levels

Material & Method

Method of Data & sample Collection

Inclusion Criteria for investigation arm

1. Patients with diagnosed Thalassemia
2. Patient age more than 2 years
3. Patients undergoing repeated blood transfusion.

Exclusion Criteria for investigation arm

1. Antenatal mother
2. Diagnosed chronic liver disease and chronic inflammatory disease.

Method of data and sample collection

1. History and clinical examination
2. Record review
3. Sample collection (both buccal smear and peripheral blood)

Method of data and sample collection

1. History and clinical examination
2. Record review
3. Sample collection

Sample Collection

Smear had been taken only from apparently healthy looking mucosa. The scrapped buccal mucosal material was then smeared onto a glass slide. This buccal mucosal smear had been taken from both study and control group subjects. Peripheral venous blood samples were withdrawn from both the study as well as control group subjects in ethylenediaminetetraacetic acid (EDTA) vial for the estimation of complete hemogram, peripheral blood smear examination. Blood samples of 5cc was taken from both the study as well as control group subjects in plain vials for the estimation of serum ferritin levels.

Study Technique

The patient who came with anemia, repeated history of infection, fever and other symptoms of thalassemia were prescribed for CBC. CBC was done from peripheral blood collected in EDTA vial. The test was done in our department in Sysmex KX-21. Patients with low hemoglobin, low MCV, MCH, MCHC and low RDW, peripheral blood smear were examined. Cases with microcytic hypochromic anemia, marked anisopoikilocytosis, presence of target cells,

reticulocytosis, presence of nucleated RBC in peripheral blood smear with or without a positive family history of thalassemia was then prescribed for High performance liquid chromatography (HPLC). In our department the HPLC machine used was BIO RAD VARIANT II. Thalassemia was diagnosed by elevated level of HbF, total absence or some presence of HbA and elevated HbA_2. After diagnosis of thalassemia was made in patient, HPLC of both parents were done to ascertain the thalassemia status among them. Our study included diagnosed thalassemia cases (by above methods) and who were on repeated blood transfusion for anemia.

Perls' method used to indicate non-heme iron in tissue such as ferritin and hemosiderin, the procedure does not stain iron that is bound to porphyrin forming heme such as hemoglobin or myoglobin. The buccal mucosal smear taken by the method described previously was then fixed immediately in 70% ethanol for 1 hour by keeping it into plastic coplin jar. Then it was stained with Perls' Prussian Blue stain. Perls' Prussian staining kit consists of potassium ferrocyanide, which would react with ferritin in the cells to form a blue colour. A fresh mixture of 1 part of 2% hydrochloric acid and 1 part of 2% potassium ferrocyanide was prepared. This mixture was then applied on the alcohol fixed buccal mucosal smear and kept for 20-30 minutes. Then the slide was rinsed by distilled water. After that the slide was counterstained by eosin and then air dried. This procedure was applied on 100 different patients buccal mucosal smear who were on repeated blood transfusion. Potassium ferrocyanide in the staining solution combines with the ferric iron forming the Prussian blue pigment.[9] The addition of hydrochloric acid increases the availability of iron within the tissue for reaction.[9] The chemical formula for the conversion of iron to Prussian blue is provided as follows

$$4FeCl_3 + 3K_4Fe(CN)_6 \text{---} Fe_4[Fe(CN)_6]_3 + 12KCL$$

↓

(Ferric ferrocyanide Or Prussian blue)

The stained slide was then examined under Light Microscope at ×100 and ×400 magnification. The presence or absence of blue colored intracytoplasmic granules in the buccal epithelial cells had observed. The presence of intracytoplasmic blue granules was considered as positive.

The peripheral blood collected was allowed to clot for serum ferritin examination. Serum was separated and stored at –20°C. Ferritin level estimation was performed by using indirect enzyme linked immunosorbent assay (ELISA) kit.

Again a portion of EDTA mixed blood was used for CBC by Sysmex KX-21 from which current hemoglobin level, MCV, MCH, MCHC and other blood parameters has been estimated along with blood smear examination under microscope.

Observation and Results

Out of 100 thalassemic patients 79 patients showed Perls' Prussian blue positivity in buccal mucosal smear. None of the 50 control subjects showed buccal mucosal positivity for Perls' Prussian blue reaction in our study.

Results

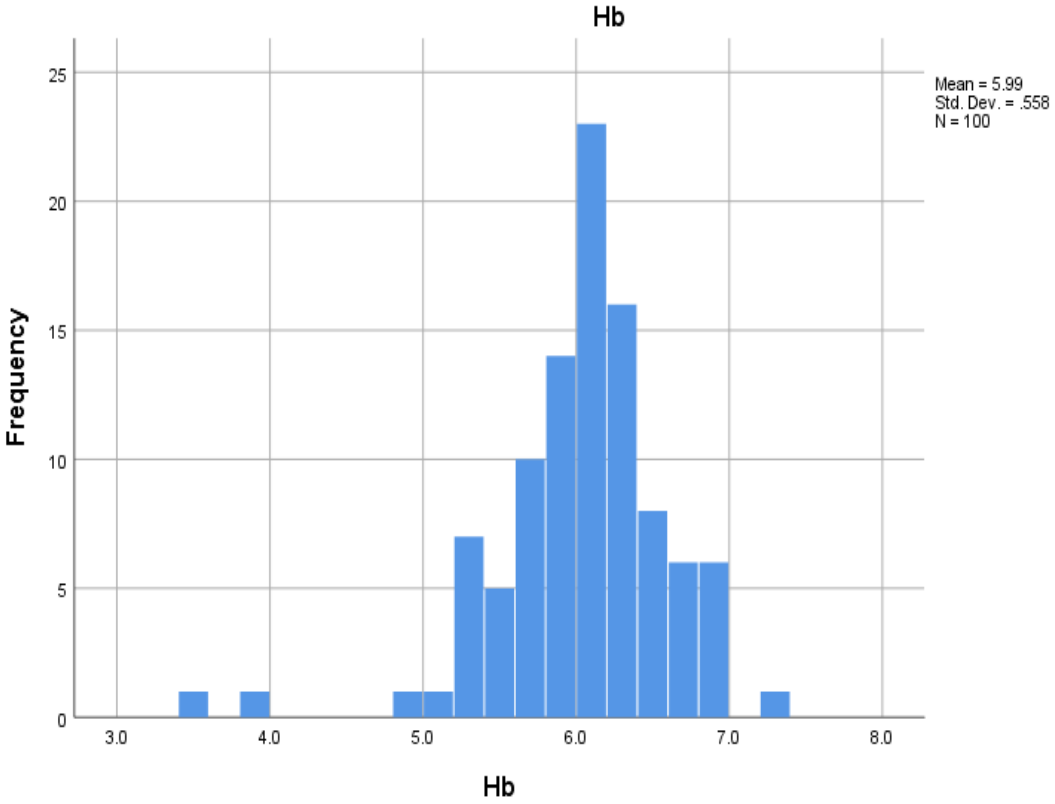
Table 1: Sex wise distribution of cases

Sex	Frequency	Percentage
Male	60	60.0
Female	40	40.0
Total	100	100.0

Table 3: Diagnosis (different types of thalassemia in the study)

Types of Thalassemia	Frequency	Percent
β thalassemia major	67	67.0
E β Thalassemia	33	33.0
Total	100	100.0

Distribution of hemoglobin level among cases

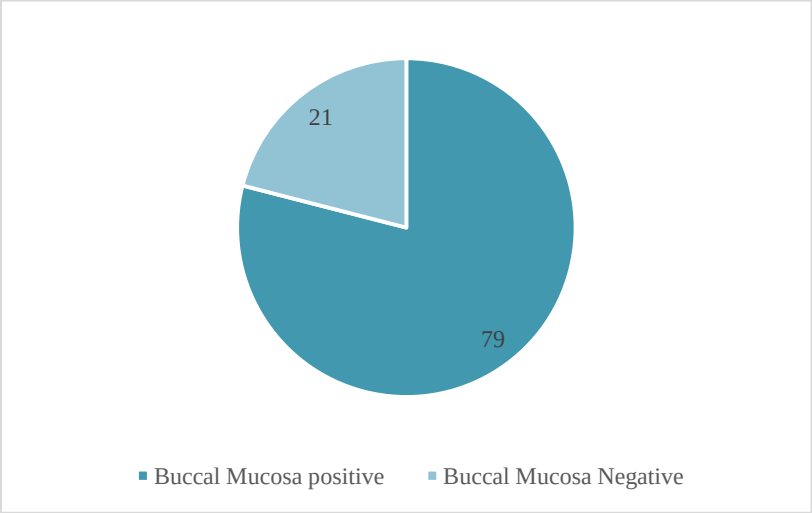


Hemoglobin level among my cases is depicted in abode diagram with Mean Hb%- 5.99 gm%, SD-.5578, Range-3.5-7.2 gm%.

Table 5: Buccal Mucosa positivity for Perls' stain among thalassemia cases

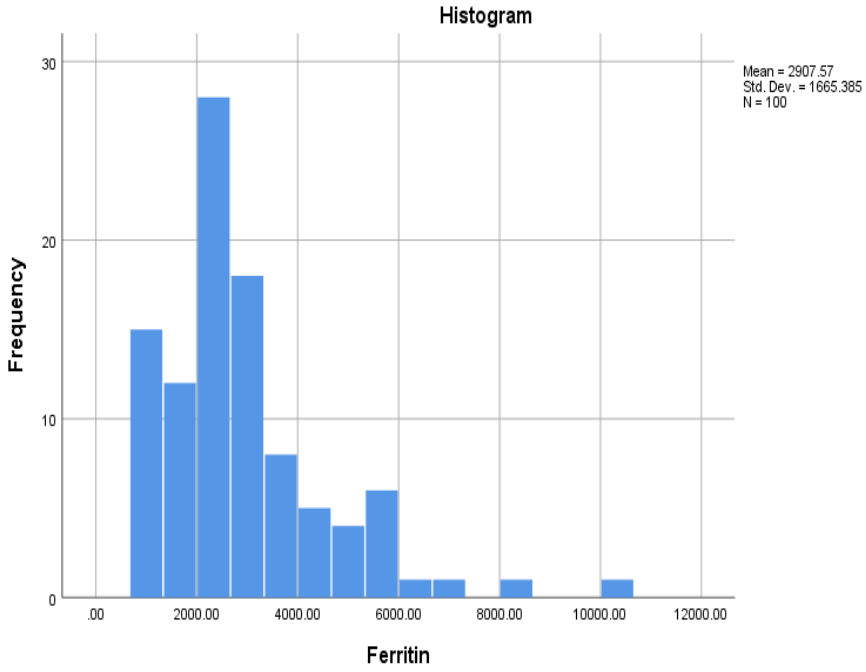
Buccal Mucosa positivity for Perls' stain among thalassemia cases	Frequency	Percent
Buccal Mucosa positive	79	79.0
Buccal Mucosa Negative	21	21.0
Total	100	100.0

Figure 5: Buccal Mucosa positivity for Perls' stain among thalassemia cases



Among 100 cases undergoing repeated blood transfusion in this study 79 cases were positive for buccal mucosa Perls' stain and 21 cases were negative for the same test.

Figure 6: Serum ferritin level distribution

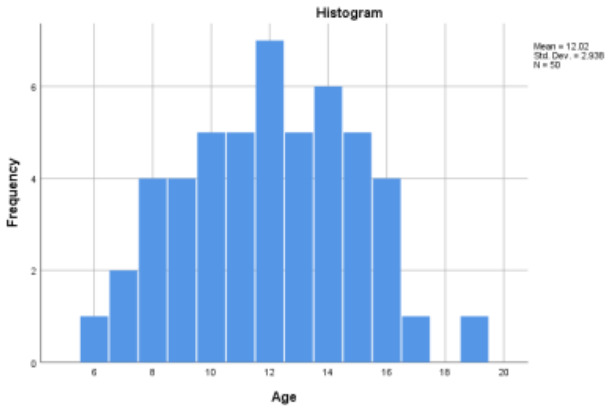


Serum ferritin level Histogram among cases - Mean serum ferritin 2907.57ng/ml, D-1665.39, Range- 727-10526 ng/ml.

Table 7: Sex wise distribution among Control

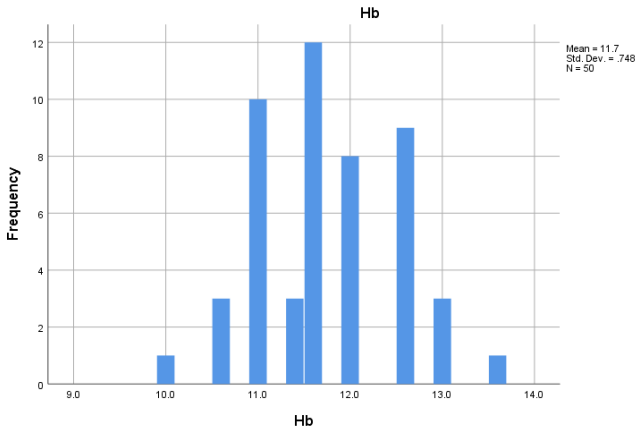
Sex	Frequency	Percent
Male	21	42.0
Female	29	58.0
Total	50	100.0

Figure 7: Sex distribution among controls



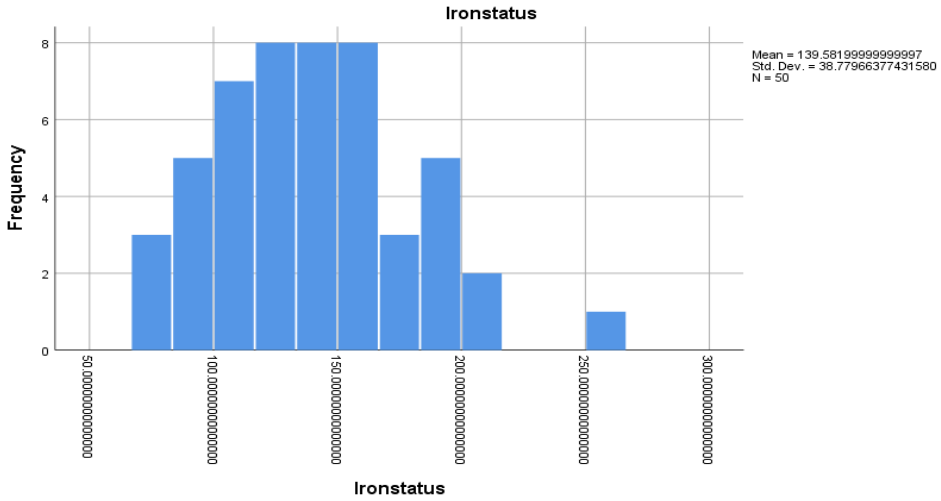
Mean Age among control in this study is12.02 with SD-2.93 and range-6-19.

Figure 9: Hemoglobin level among 50 controls



Mean Hemoglobin level among 50 controls were Hb% 11.696gm%, SD-.7480, Range10-13.5gm%.

Figure 10: Serum ferritin level among 50 controls



Mean Serum ferritin level among 50 controls is 139.58ng/ml with SD-38.77 and range- 69-260ng/ml.

Table 8: Buccal mucosal Perls' stain test results among controls

Group	No	Positive	Negative	Percent
Controls	50	0	50	100%

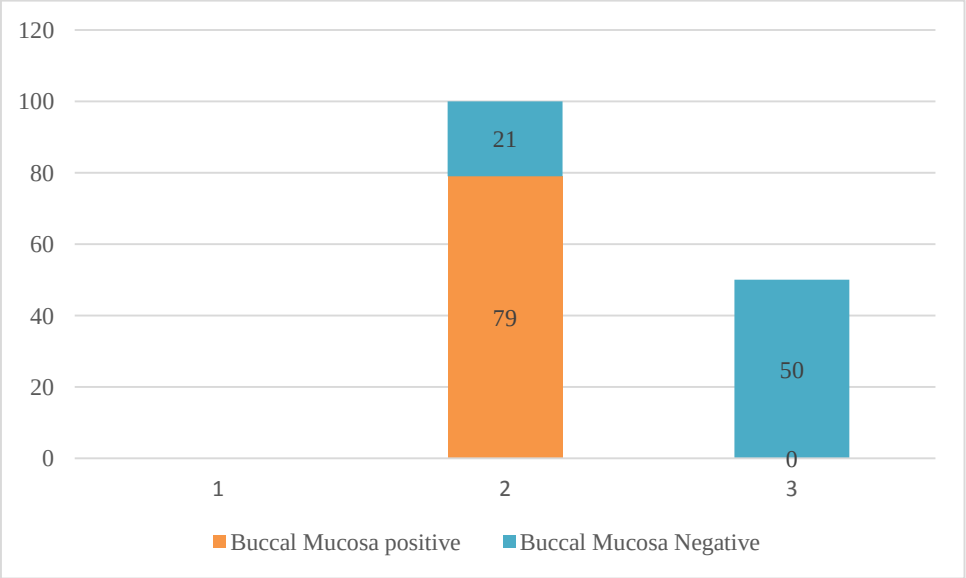
100% test negativity of buccal mucosal Perls' stain among controls test results were found.

Association

Table 9: Buccal mucosa Perls' stain positivity status: Case versus control

	Case & control		Total	Chi Square (Fisher's Exact value)	P value
	Case	Control			
Buccal Mucosa positive positive posit positive	79	0	79	83.451	<0.001
Buccal Mucosa negative aNegative	21	50	71		
Total	100	50	150		

Figure 9



Among 100 thalassemia patients undergoing repeated blood transfusion and all with high serum ferritin level, 79 were positive for buccal mucosal iron deposition test (Perl's Prussian Blue test) and 21 were negative. Again among 50 controls all are negative for Perls' stain of buccal mucosa. Applying the Chi Square test the value of the test is 83.451 and the p value is <0.001 which is significant.

Table 10: Differences of mean Serum ferritin level among cases and control (Unpaired t test)

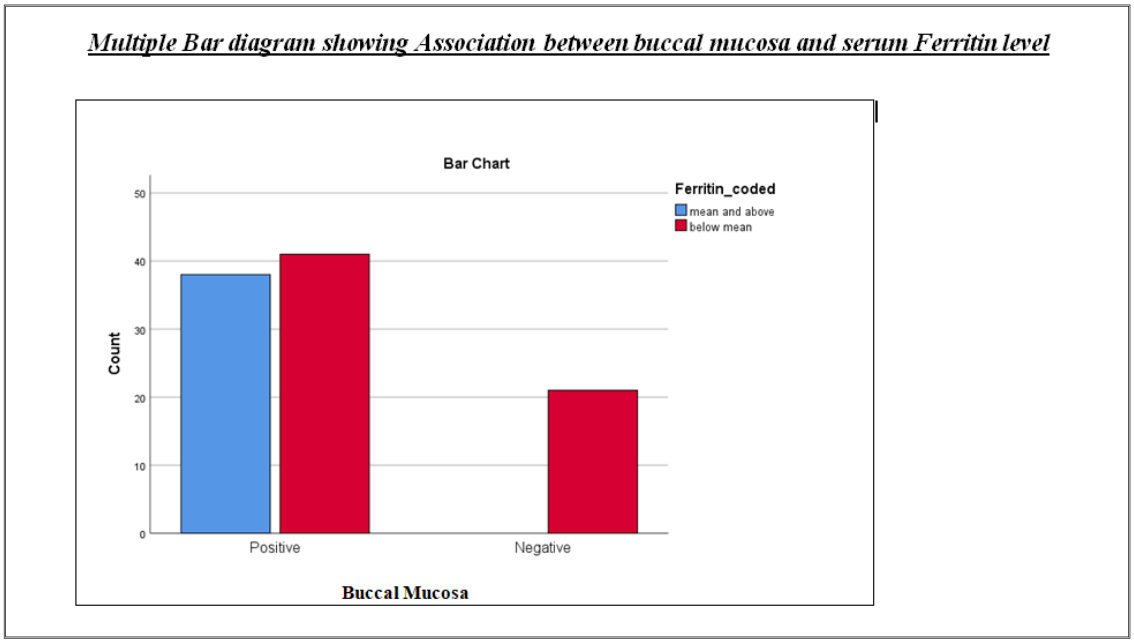
Case / Control	Frequency	Mean Serum Ferritin level	P value
Cases	100	2907.57	<0.001
Control	50	139.58	

The mean serum ferritin among cases was 2907.57ng/ml and that among control was 139.58ng/ml. Applying the unpaired t test the p value of this test was <0.001 which was significant.

Table 11: Association between serum ferritin and buccal mucosal Perls' stain status among cases

	Buccal Mucosa positive	Buccal Mucosa Negative	Total	Chi Square (Fisher's Exact value)	P value
Serum ferritin level mean and above mean	38	0	38	16.292	<0.001
serum ferritin level below mean	41	21	62		
Total	79	21	100		

Figure 11: Multiple Bar diagram showing Association between buccal mucosa and serum Ferritin level



In this study considering the mean serum ferritin level as a cut off value, the patients having serum ferritin level of mean value and above mean value showed 100% (38 out of 38) buccal mucosal positivity for perls' stain and patients having ferritin level below mean value showed 66.12% (41 out of 62 positive) buccal mucosal positivity. The Chi Square (Fisher's) exact value was 16.292 and the association was found significant ($p < 0.001$).

Table 12: Correlation between buccal mucosal Perls' stain positivity and serum ferritin level

Serum ferritin	Pearl's Prussian Blue staining positivity
Correlation co efficient	0.542
p value	<0.001
N	100

The correlation between serum ferritin level and buccal mucosal Perls' stain positivity was done, the correlation co-efficient was 0.542 and it was found significant (p value <0.001).

Discussion

The severe and most frequently encountered hemoglobinopathies in India include the thalassemias. The age of the patients in our study ranged from 3-38 years with a mean age 13.12 ±5.97years.

Our study has shown almost an equal incidence of disease in both sexes with slightly more preponderance in male sex. Out of randomly selected cases maintaining the inclusion and exclusion criteria, my study includes 60 male patients and 40 female patients. This may be a random selection bias or may be due to the male preponderance seen in India which has been attributed to a gender bias, rather than an actual preponderance of disease in male. The existence of female disadvantage in large parts of the country, especially the northern states, has been clearly identified. Studies across India have found that boys are much more likely than girls to be taken to a health facility when sick.[5]

Among the 100 cases of thalassemia taken into my study, the different subtypes were found as β-Thalassemia Major 67%, E β- thalassemia 33%, who are undergoing repeated blood transfusions over a significant period, atleast received 10 transfusions.

The decision to start regular transfusions is clear when the initial hemoglobin level is well below 7 g/dL.[5] To assess a child's need for routine transfusions due to thalassemia, anemia caused by sepsis, viral infection, folic acid deficiency must be ruled out. Assessment may be accomplished by withholding transfusions and monitoring weekly hemoglobin level. If the hemoglobin drops under 7 g/dL on two occasions, two weeks apart, then regular transfusions should be commenced.[,5] Patients with a hemoglobin level more than 7 g/dL may sometimes require regular transfusions in the presence of growth impairment, marked skeletal changes or extramedullary hematopoiesis. Thalassemic patients should undergo regular blood transfusions one to two times in a month to maintain a hemoglobin levels of 9-10 g/dL.[5] In my study the measured mean Haemoglobin percentage (Hb%) was found 5.99, with standard deviation (SD) 0.5578 and Range of 3.5-7.2 gm%, which falls far from the desired level and can be due to late presentation with severe anemia, irregular attendance for blood transfusion, nutritional deficiency, parasitic infestation in lower socio-economic group in our country.

In this study assessment of serum ferritin level was taken as an indicator of iron overload in thalassemia patients, undergoing repeated blood transfusion. The Mean Serum Ferritin of my study was 2907.57ng/ml, with SD-1665.39, Range- 727-10526 ng/ml which correlates with all previous studies on serum ferritin level in thalassemia patients undergoing repeated blood transfusion.[6,11]

Perls' Prussian blue reaction is considered as the first classical histochemical reaction applied in the field of hematology for iron staining. In our study, exfoliated cells from the buccal mucosa of 79 of the 100 thalassemic patients (79%) group revealed positivity for Perls' Prussian blue reaction. My observation results of Perl's Prussian blue staining positivity were higher than the studies of Nandprasad et al. (2010) who observed 65% Perl's positivity (65 out of 100 patients), Bhat et al. (2013) who reported 71.7% positivity (43 patients positive out of 60), Chittamsetty et al. (2013) who observed 72.5% (29 out of 40 β-thalassemia major patients) and Gupta et al. (2014) who observed 61.6% Perl's positivity (37 out of 60 cases).[8,11,12,13] But our study result in this aspect showed a lower positivity to that of the study by Gururaj and Sundharam et al. (2004) who reported 100% Perl's positivity in the 10 patients that they examined and ajit Singh Rathore et al who recorded 82.9% positivity.[14,15] Further, I observed that none of my control subjects showed Perls' Prussian blue positivity which also correlates with the previous studies described.

Again in our study the buccal mucosal positivity for Perls' stain correlates significantly with serum ferritin level (p value <0.001) among these patients, which also is similar to the previous study done by Atul A. Bhatt et al. among thalassemia major patients.[11] Considering the mean serum ferritin level as a cut off value the patients having serum ferritin level equal or above mean showed 100% buccal mucosal positivity whereas patients having serum ferritin level below mean showed 66.12% buccal mucosal positivity for Perls' stain. So it can be stated that increase in serum ferritin level significantly associated with increase in chances of positivity of buccal mucosal stain.

The serum ferritin levels were significantly higher in our study group as compared to control group. This rise in serum ferritin levels in the study group is in accordance with those reported by various authors. The mean serum ferritin levels reported in previous studies were 3820 ng/mL by Silvilairat et al.[16] and 3390 ng/mL by Ikram et al.[6] in thalassemia patients undergoing blood transfusion. It has observed from my study and also from various other authors that serum ferritin is markedly elevated in thalassemic patients undergoing repeated blood transfusion and a great variation in serum ferritin levels has been observed in these patients. This may be related to various factors such as age of patient, age of onset of blood transfusions, number and regularity of

blood transfusions, severity of disease, use of chelation therapy, whether taking the chelation therapy regularly, socioeconomic status, nutritional deficiencies and other systemic and metabolic factors.

The exact reason for 100% non-positivity of Perls' Prussian blue staining in our study may be attributed to the difference in the total sample size, age of the patients of various studies, varying mean serum ferritin levels of the respective patients in the different studies and last but not the least a hypothetical correlation can be done in light of iron metabolism.

The excess amount of iron in the blood gets accumulated in various tissues in our body. It may depend upon various factors including the formation of iron storage pool. As the amount of hemosiderin forms in different tissue varies, in the same way the amount of apoferritin and therefore, ferritin formed in exfoliated buccal cells, may vary. This may invariably affect the Perl's positivity. Again, in case of transfusional iron overload, iron level increases due to destruction of erythrocytes and this iron accumulates first in reticuloendothelial macrophages, which only after that spills over into parenchymal cells.[17]

Again, ferritin cannot be visualized always under light microscope despite of its presence within the cells and in those cases it can be observed by electron microscope only. Hemosiderin, can be visualized as a blue coloured intracytoplasmic granules under light microscope, due to its larger size. Hemosiderin is thought to be formed when the quantity of iron exceeds the apoferritin storage pool, as the ferritin molecule ages [18].

Conclusion

To conclude, I ascertained the correlation of Perls' Prussian positivity of buccal mucosal epithelial cells to serum ferritin. Our results showed a positive correlation. So it can be said that the positive staining in exfoliated buccal cells of these patients may give a clue to increased serum ferritin levels, which can be used as a screening tool in peripheral and rural setup where serum ferritin level estimation is not readily available. Moreover it is a simple, cheap, bloodless, painless, quick and easy technique that can be comfortably done at peripheral setups without any risks, to demonstrate iron overload in the body tissues. Again as it is a non-invasive procedure it can be done frequently if needed. So in our opinion this method may be used as a qualitative test to ascertain iron overload in thalassemia patients undergoing repeated blood transfusion.

Reference

1. Modell B, Darlison M. Global epidemiology of haemoglobin disorders and derived service indicators. Bull World Health Organ. 2008;86(6):480-487. doi:10.2471/blt.06.036673
2. Trehan A, Sharma N, Das R, Bansal D, Marwaha RK. Clinicoinvestigational and demographic profile of children with thalassemia major. Indian J Hematol Blood Transfus. 2015;31(1):121-126. doi:10.1007/s12288-014-0388-y
3. Weatherall DJ, Clegg B (eds) The thalassemia syndromes, 4th edn. Blackwell Sciences, Oxford, pp 133–191, 288-289
4. Verma IC, Saxena R, Kohli S (2011) Past, present and future scenario of thalassemic care and control in India. Indian J Med Res 34:507–521
5. Cappellini MD, Cohen A Porte,r J Taher A, Viprakasit V. GUIDELINES FOR THE MANAGEMENT OF TRANSFUSION DEPENDENT THALASSAEMIA (TDT); 3rd Ed:p 15-19, 21-22, 28-34
6. Ikram N, Hassan K, Younas M, Amanat S. Ferritin levels in patients of beta thalassemia major. Int J Pathol. 2004; 2:71–4.
7. Angelucci E, Brittenham GM, McLaren CE, Ripalti M, Baronciani D, Giardini C, et al. Hepatic iron concentration and total body iron stores in thalassemia major. N Engl J Med. 2000;343:327–31.

8. Gupta S, Trichal V, Malik R, Nigam R, Choudhary R, Shrivastava A. Perls' prussian blue positivity in exfoliated buccal cells of b thalessemia major patients and its correlation with serum ferritin. J Evol Med Dent Sci. 2014;3:5135–40.
9. Churukian CJ. Pigments and minerals. In: Bancroft JD, Gamble M, editors. Theory and practice of histological techniques. 6th ed. China: Churchill Livingstone Elsevier Ltd; 2008. pp. 233–6.
10. Mohanty D, Colah RB, Gorakshakar AC, et al. – Prevalence of beta-thalassemia and other haemoglobinopathies in six cities in India: a multicentre study. J Community Genet 2013; 4:33-42
11. Bhat AA, Parwani RN, Wanjari SP. Demonstration of iron in exfoliated buccal cells of β-thalassemia major patients. J Cytol. 2013;30:169–73
12. S Nandaprasad, P Sharada, M Vidya, et al.;Oral Exfoliative Cytology In Beta Thalassaemia Patients Undergoing Repeated Blood Transfusions; The Internet Journal of Pathology. 2008 Volume 10 Number 1.
13. Chittamsetty H, Munishekar Syed, Ahmed Afroz, Palla Churu Suri Sridevi, et al. 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
14. Gururaj N, Sivapathasundaram B. Demonstration of iron in the exfoliated cells of oral mucosa. J Oral Maxillofac Pathol. 2003;7:37-9
15. Rathore AS, et al. Oral exfoliative cytology as a screening tool for iron overload in β-thalassemia patients; Int J Appl Basic Med Res.2016;6(1):28-30
16. Silvilairat S, Sittiwangkul R, Pongprot Y, Charoenkwan P, Phornphutkul C. Tissue Doppler echocardiography reliably reflects severity of iron overload in pediatric patients with beta thalassemia. Eur J Echocardiogr. 2008;9:368–72.
17. Andrews NC. Disorders of iron metabolism. N Engl J Med. 1999; 341:1986–95.
18. Guyton AC, Hall JE. Guyton & Hall Textbook of physiology. 9th ed. New Delhi India: Prism Books Pvt. Ltd; 1996. Red blood cells, anemia and polycythemia; pp. 353–71.