Buccal Mucosa Exfoliative Cell Prussian Blue Stain Co-Relates with Iron Overload in β -Thalassemia Major Patients

Pooja K. Gajaria^{1,3} · Ujwala M. Maheshwari¹ · D. B. Borkar¹ · Reeta Dhar¹ · Varsha Pancholi²

Received: 20 July 2016 / Accepted: 7 March 2017 / Published online: 21 March 2017
© Indian Society of Haematology & Transfusion Medicine 2017

Abstract Thalassemics require regular blood transfusion therapy leading to iron overload in the body tissues, which is a major cause of morbidity and mortality in these patients. We hereby attempted to measure this iron overload by means of exfoliative cytology, a non-invasive and inexpensive technique. The aims and objectives of our study were: 1. To detect iron overload by oral exfoliative cytology using Perl's Prussian blue stain in β -thalassemia major patients. 2. To correlate staining positivity with serum ferritin levels. Smears were obtained from buccal mucosa of 50 β -thalassemia major patients (who had taken more than 12 transfusions) and 25 healthy subjects of the same age group as controls. Smears were stained with Perl's Prussian blue. Blood samples were taken from the study group for estimation of serum ferritin levels. Grading criteria were defined for assessing the Prussian blue positivity. Perl's positivity was observed in 49 out of 50 of thalassemic patients (98%). 1 patient had Grade 0, 7 patients had Grade I, 5 had Grade II, 12 had Grade III, 14 had Grade IV while 11 patients had Grade V positivity. Spearman Rank's Correlation Co-efficient was 0.38, signifying a weak positive correlation between positivity of buccal smears for Perl's Prussian blue staining and respective 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 as well as diagnostic tool. With the grading system we can give a semi-quantitative assessment of the same.

Keywords β Thalassemia major · Perl's Prussian blue · Serum ferritin · Buccal mucosa · Hemosiderosis

Introduction

β -thalassemia major also known as Cooley's anaemia is characterised by severely reduced or absent production of β globin chains of the normal human hemoglobin. The recommended treatment for thalassaemia major involves life-long regular blood transfusions, usually administered every two to five weeks, to maintain the hemoglobin level above 9–10.5 g/dl. Long term transfusion has its multiple complications, infection and iron overload being the dreadful ones. Post-transfusion iron overload is a major cause of morbidity and mortality in β -thalassemia major patients. Hemosiderin deposits in the heart, liver, endocrine glands and lungs of these affected patients [1]. Serum iron, serum ferritin estimation, transferrin saturation, liver biopsy, T2* Magnetic Resonance Imaging (MRI) of the liver and heart are all being used to monitor iron overload status and effect of iron chelation therapy in these patients [2, 3]. With this study, we attempted to measure this iron overload by means of exfoliative cytology, a noninvasive and inexpensive technique.

Aims and Objectives

1. To detect and grade iron overload by oral exfoliative cytology using Perl's Prussian Blue stain in β -thalassemia major patients.

✉ Pooja K. Gajaria
drpoojakg@gmail.com

¹ Department of Pathology, Mahatma Gandhi Mission's Medical College and Hospital, Navi Mumbai, India

² Triumph Blood Bank and Thalassemia Day Care Centre, Navi Mumbai, India

³ 1401/1402, Shobha Suman CHS, M. M. Malaviya Road, Opp. Telephone Exchange, Mulund (West), Mumbai 400 080, India

2. To correlate Perl's Prussian Blue staining positivity with serum ferritin levels.
3. To determine whether this technique can be used as a screening and diagnostic tool for detection of iron overload in these patients.

Materials and Methods

The prospective study was conducted from February 2014 to July 2015, after approval from Institution Ethics Review Committee. 50 diagnosed cases of β -thalassemia major (after High Pressure Liquid Chromatography), who had taken more than 12 transfusions were included in the study. A brief history regarding initiation of iron chelation therapy was obtained. 25 healthy subjects of the same age group were chosen as controls.

Subjects were asked to rinse their mouth with distilled water. Scrapings were taken from the buccal mucosa (of both the study and the control group) with the help of CytoBrush. Smears were spread on clean, dry glass slides and fixed immediately in 90% methanol for 1 h followed by Perl's Prussian Blue staining. Smears were evaluated for positivity under the light microscope.

Under all aseptic precautions, venous blood was collected in yellow topped SST gel vacutainer from the study group. Serum ferritin levels were estimated using the principle of electrochemiluminescence on the Cobas e411 analyzer (Roche diagnostics).

A *grading system* was devised by us as an attempt to provide a semi-quantitative assessment of the Perl's Prussian blue staining positivity. We used the Olympus 21i microscope for assessment of all our smears (eyepiece— $\times 10$; diameter 20 mm). First, the slide was screened at low power objective lens ($\times 10$) to judge the cellularity and to look for the presence or absence of *blue coloured intracytoplasmic* iron granules. Then, 5 high power fields (objective lens, $\times 40$) fulfilling the following 3 criteria were chosen for grading purpose:

1. Evenly dispersed cells with minimal overlapping.
2. Minimum 20 squamous cells and maximum 30 cells per high power field ($\times 40$).
3. Presence/Absence of blue intracytoplasmic granules.

Grading:

Grade 0—No granules

Grade I— <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 <3 high power field

Grade V—Coarse granules or clumps seen in $3/ > 3$ high power field

An average of findings in 5 high power fields ($\times 40$) was taken and accordingly, appropriate grade assigned.

Results

Out of the 50 β -thalassemia major patients, 32 were males (64%) and 18 were females (36%). In the control group, 18 were males (72%) and 7 were females (28%). The age group of the study group ranged from 1.5 to 22 years with a mean of 7.99 years. The maximum cases were up to 5 years of age (23 cases) while there were only 2 cases in the group of 21–25 years. 41 patients were on iron chelation therapy (82%) and only 9 patients were yet not started on chelation (18%). The mean $\pm$ SD serum ferritin level of the study group was 2825.696 ± 1507.548 ng/ml. 14 out of 50 patients in the study group (28%) had serum ferritin levels in the range 2201–3200 ng/ml and only 1 patient (2%) had levels above 6200 ng/ml.

49 patients (98%) out of 50 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, 14 out of 50 (28%) had Grade IV positivity, followed by 12 patients having Grade III (24%) while only 1 patient (2%) was negative belonging to Grade 0 (Figs. 1, 2, 3, 4, 5, 6). All 25 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 significant at 1% level of significance (p value < 0.001).

In order to correlate the buccal smear positivity with serum ferritin levels, Spearman Rank's correlation coefficient was calculated. It was 0.38 implying a weak positive correlation between the two.

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 (Table 1).

Discussion

Transfusion hemosiderosis is a major cause of morbidity and mortality in β -thalassemia major. Though newer modalities of treatment to chelate the iron are becoming available yet the detection and quantitation of iron overload remains a problem mainly due to invasive and expensive nature of the available tests.

Fig. 1 Perl's Prussian Blue staining of squamous epithelial cells from buccal mucosa showing absence of blue granules—Grade 0 or Negative ($\times 400$). Counterstain used—Eosin (colour figure online)

Fig. 2 Perl's Prussian Blue staining of squamous epithelial cells from buccal mucosa showing <5 intracytoplasmic blue granules—Grade I Positivity (Yellow circle in both *a* and *b*) ($\times 1000$). Counterstain used—Eosin (colour figure online)

Fig. 3 Perl's Prussian Blue staining of squamous epithelial cells from buccal mucosa showing 5–10 intracytoplasmic blue granules—Grade II Positivity (Yellow circle in *a* at $\times 1000$ and Green arrows in *b* at $\times 400$). Counterstain used—Eosin (colour figure online)

Staining of exfoliated cells from buccal mucosa with Perl's Prussian blue can be adopted as a cheaper, yet accurate diagnostic approach to detect the iron overload. Through our study we have provided the criteria to grade the smears providing a semi-quantitative assessment of the same.

Our results of Perl's Prussian blue staining positivity were similar to Gururaj and Sundharam et al. (2004) who reported 100% Perl's positivity in the 10 patients that they examined [4]. These results, however, were higher than that 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) [5–8]. 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.

Fig. 4 Perl's Prussian Blue staining of squamous epithelial cells from buccal mucosa showing 10–20 intracytoplasmic blue granules—Grade III Positivity (Green arrows in *a* and Green circle in *b*) ($\times 1000$). Counterstain used—Eosin (colour figure online)

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 the same. The controls were healthy and did not suffer from iron overload explaining their negative results.

The single patient with negative Perl's Prussian blue staining had serum ferritin level of 2124 ng/ml. The negative reaction, even in spite of high serum ferritin levels in this patient 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

Fig. 5 Perl's Prussian Blue staining of squamous epithelial cells from buccal mucosa showing >20 intracytoplasmic blue granules—Grade IV Positivity (Yellow arrows and circles in *a* at $\times 400$ and blue clumps shown by Green arrows in *b* at $\times 1000$). Counterstain used—Eosin (colour figure online)

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. When hemosiderosis occurs, iron accumulates in reticuloendothelial macrophages first and only later spills over into parenchymal cells [9].

Value of Spearman Rank's correlation coefficient of 0.38, in our study was similar to Bhat et al. whose correlation coefficient was 0.424 while it was lower than that of Gupta et al. who had a correlation coefficient of 0.76 [6, 8]. The studies by Gururaj et al. and Nandprasad et al., however did not compare the presence of blue granules in exfoliated cells of buccal mucosa with serum ferritin levels [4, 5].

This is the first study which has provided a grading system for buccal smear Perl's staining in order to provide a semi-quantitative assessment of the same. Moreover, it was observed that the grade of buccal smear positivity

Table 1 Comparison of buccal smear grades with the respective mean serum ferritin levels of the study group

Buccal smear Perl's Prussian blue staining grade	Number of patients (%)	Serum ferritin mean (ng/ml)	Serum ferritin SD (ng/ml)	Serum ferritin SEM (ng/ml)	F-stat	<i>p</i> value
0	1 (2)	2124.000	–	–	2.51	<0.05*
I	7 (14)	2124.814	817.645	309.041		
II	5 (10)	2178.600	944.244	422.279		
III	12 (24)	2665.958	787.944	227.460		
IV	14 (28)	2598.700	1550.889	414.492		
V	11 (22)	4092.800	2024.193	610.317		

* Significant at 5% level of significance

Fig. 6 Perl's Prussian Blue staining of squamous epithelial cells from buccal mucosa showing intracytoplasmic blue coarse granules and clumps obscuring the cells—Grade V Positivity ($\times 1000$). Counterstain used—Eosin (colour figure online)

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. and Chittamsetty et al. had included the patients with 15 or more number of blood transfusions in their study [5, 7]. In our study, Perl's Prussian blue positivity was observed even with the number of transfusions being 12, implying that this test can be used as a screening modality also, besides regular monitoring.

Most of these patients (82%) were on prolonged 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.

However, we could not compare the Perl's Prussian Blue positivity with liver biopsy, T2* MRI of liver/heart, MUGA scan (Multigated acquisition), liver dry weight iron because of the expensive and invasive nature of these tests. More studies therefore need to be conducted comparing exfoliative cytology with these tests. Also a co-relation of this test with clinical manifestation of iron overload in various organs needs to be done.

Conclusion

We, therefore conclude that Perl's Prussian Blue staining of exfoliated cells from buccal mucosa can not only be used to assess iron overload in β -thalassemia major patients, but can also be used 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, regular monitoring of overload by serum ferritin can be replaced by exfoliative cytology of buccal cells which is easy to obtain and non-invasive. The group of affected patients comprises mostly of the paediatric age, so invasive, painful and expensive tests, can thus be avoided.

Acknowledgements We are grateful to the staff of Triumph Blood Bank and Thalassemia Day Care Centre and would also like to acknowledge the hard work of our senior technician Mr. Laxman Ghadi for his consistent help.

Compliance with Ethical Standards

Conflict of interest We declare that we have no conflicts of interest.

Human and Animal Rights All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional research committee.

Informed Consent Informed consent was obtained from all individual patients/their guardians, included in the study.

References

1. Greer JP, Arber DA, Glader B, List AF, Means RT, Frixos P, Rodgers GM (2014) Wintrobe's Clinical Hematology, 13th edn. Wolters Kluwer Lippincott Williams and Wilkins Health
2. Brittenham G, Cohen A, McLaren C, Martin M, Griffith P, Nienhuis A et al (1993) Hepatic iron stores and plasma ferritin concentration in patients with sickle cell anemia and thalassemia major. *Am J Hematol* 42(1):81–85
3. Ghugre N, Enriquez C, Gonzalez I, Nelson M, Coates T, Wood J (2006) MRI detects myocardial iron in the human heart. *Magn Reson Med* 56(3):681–686
4. Gururaj N, Sundharam S (2003) Demonstration of iron in the exfoliated cells of the oral mucosa. *J Oral Maxillofac Pathol* 1:37–39
5. Nandprasad S, Sharada P, Vidya M, Karkera B, Hemanth M, Prakash N (2009) Oral exfoliative cytology in beta thalassemia patients undergoing repeated blood transfusions. *Internet J Pathol* 10:7
6. Bhat Atul A, Parwani RK, Wanjari SP (2013) Demonstration of Iron in exfoliated buccal cells of β thalassemia major Patients. *J Cytol* 30(3):169–173
7. Chittamsetty H, Munishekar Syed, Ahmed Afroz, Palla Churu Suri Sridevi et al (2013) A Non- invasive Technique which demonstrate iron in the buccal mucosa of sickle cell anaemia and thalassemia patients who undergo repeated blood transfusion. *J Clin Diag Res* 7(6):1219–1222
8. Gupta S, Trichal VK, Malik R, Nigam RK, Choudhary R, Shrivastava A (2014) Perls' prussian blue positivity in exfoliated buccal cells of β thalassemia major patients and its correlation with serum ferritin. *J Evol Med Dental Sci* 3(19):5135–5140
9. Andrews NC (1999) Disorders of iron metabolism. *N Engl J Med* 341:1986–1995