ORIGINAL PAPER

VALIDITY OF USING ORAL MUCOSAL EXFOLIATIVE SMEAR AS A SCREENING TOOL OF IRON OVERLOAD IN β-THALASSEMIA MAJOR PATIENTS

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ABSTRACT

Introduction: Iron overload is one of the most common life-threatening disorders among thalassemia patients. Iron level is usually monitored using serum ferritin level or chemical assessment of liver biopsy. However, these tests are extremely invasive, especially on repeated use.

Objectives: This study aims to evaluate the validity of using oral mucosal exfoliative smear stained with Perls' Prussian blue reaction as a tool in qualitative evaluation of iron overload, and assessing the possibility of using cellular expression of Perls' Prussian blue-stained cells as a tool in quantitative assessment of serum ferritin level in β-thalassemia major patients.

Material and methods: Buccal mucosal scrapings were taken from 69 β-thalassemia major patients (40 males, 29 females), aged 5 to 33 years old (average, 15.4 years), who have undergone blood transfusion for more than 15 times. The scrapings were fixed with methanol (99.8%) and stained with acidified potassium ferrocyanide solution, followed by Gram staining (safranin staining). The scrapings were evaluated using photomicrograph at 40 × 10 magnification.

Results: Among members of the sample, 59 patients (87%) were positive for Perls' Prussian blue reaction. A moderate positive correlation was found between positivity of Perls' Prussian blue reaction and serum ferritin level. A moderate positive correlation was also found between expression of iron within oral mucosal exfoliative cells and serum ferritin level.

Conclusions: Oral mucosal exfoliative smear can be used in both qualitative and quantitative evaluation of iron overload.

Key words: oral mucosa, ferritin, iron overload, β-thalassemia, Perls'-Prussian blue reaction.

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INTRODUCTION

Thalassemia is one of the most common monogenic disorders that affect the manufacturing of hemoglobin. Hemoglobin is the main protein that exists within red blood cells (RBCs). It is responsible of binding and carrying oxygen particles from the lungs to the various body tissues [1]. The majority of β-thalassemia cases exist within parts of sub-Saharan Africa, across the Middle East, and the Indian subcontinent [2]. People carrying the recessive gene usually include 20-25% of population from a certain region. However, some exceptional cases are higher than this number [3].

Each hemoglobin particle consists of two chains of both α and β globin. Each one of these globin chains are connected to a heme particle [4]. Some affected individu-

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als might be homozygous or heterozygous regarding genes responsible for manufacturing of α and β chains. Heterozygous individuals have α or β-thalassemia minor, which has less severe symptoms. Meanwhile, homozygous individuals are known as α or β-thalassemia major (Cooley's anemia), which presents with much more severe symptoms [5]. Cases with β-thalassemia major show severe impairment in the production of β globin gene sequence, which leads to anemia. RBCs demonstrate microcytic and hypochromic appearance with an aberrant morphology [6].

β-thalassemia patients undergo repeated blood transfusions treatment. This treatment leads to iron overload, which is considered one of the most common life-threatening disorder among thalassemia patients [7].

Minerals are important non-organic elements that are necessary for all body cells in order to maintain regulatory functions of the body. There are around 4.5 g of iron within the average adult male body, most of which are concentrated within hemoglobin particles and within heme-containing proteins [8]. Under normal circumstances, iron is absorbed by the human body at an average of 1 mg daily. Absorbed iron is distributed for storage, transportation, and enzymatic functions as ionic compounds in the body tissues [9].

Iron overload is one of the most common disorders among β-thalassemia patients. The levels of iron within the body cells depend on the frequency of blood transfusion and the effectiveness of iron chelation therapy [10]. Iron overload within parenchyma cells can be evaluated using liver or bone marrow biopsy. Quantitative evaluation using liver biopsy is considered the gold standard to estimate iron level within the body. However, using a single biopsy in assessing long-range effects of repeated blood transfusions provides limited results. Therefore, repeated biopsies should be undertaken in order to obtain reliable results [11].

Perls' Prussian blue staining is an effective test for the evaluation of iron within cells. In addition, it distinguishes iron from other hepatocellular cytoplasmic pigments [12]. Perls' Prussian blue reaction is based on the principle acidified potassium ferrocyanide solution binds to iron in the tissues, forming an insoluble blue-purple precipitate [13]. However, these procedures are invasive and are not advisable in every case.

Oral exfoliated cells can be used in the assessment of both quantitative and qualitative pathologic alterations, which are associated with the parent tissue [14]. It is a quick, non-invasive, and relatively low-cost technique, especially when compared to other methods. Therefore, this principles are applied to demonstrate the iron in oral exfoliated cells of the iron overloaded patients using the Perls' Prussian blue reaction.

OBJECTIVES

The aim of our study was to evaluate the validity of using oral mucosal exfoliative smear stained with Perls' Prussian blue reaction as a tool in qualitative evaluation of iron overload. Additionally, an estimation of the possibility of using cellular expression of Perls' Prussian blue stained cells as a tool in quantitative assessment of serum ferritin level in β-thalassemia major patients was performed.

MATERIAL AND METHODS

A total of 69 patients (44 males and 25 females), aged 5 to 33 years old (average, 15.4 years) from the Department of Thalassemia, General Medical Clinics, Damascus, Syria, who were selected through a convenience randomized sampling technique, were enrolled into the study. An informed consent from every patient or legal guardian was obtained prior the study. All included patients were diagnosed with β-thalassemia major, had more than 15 blood transfusions, and no other developmental or hereditary disorders.

Each patient from the study group was asked to gargle with distilled water. A wooden spatula was prepared and moistened using normal saline. The scraps were obtained using a gentle scraping move of the wooden spatula on normal looking buccal mucosa while exerting slight pressure. The scraps were smeared onto the center of clean, fresh, and dry glass slides, and spread over a large area to prevent clumping of the cells. The slides were immediately immersed with pure 99.8% methanol for at least one hour to ensure adequate fixation of the cells.

Each slide was stained using acidified potassium ferrocyanide solution, which would react to the ferritin in the cells giving blue colored non-soluble compound (Perls' Prussian blue). The slides were counterstained using Gram stain (safranin), which stain the cells with pink color and the nuclei with red color.

The slides were evaluated using a light microscope at 400 × magnification, and random 5 fields were selected to evaluate the presence or absence of blue granules in the cells, which indicate the presence of iron overload. A score of 0 to 4 was given to each slide to assess the expression of iron granules within the cells, based on the estimation of the amount of blue granules within mucosal cells (Figure 1). A score of 0 meant a negative case with no visible blue granules, while a score of 4 meant a positive case with a high number of granules visible within the cells. This analysis was performed by two independent observers in order to minimize the bias caused by the subjectivity of such assessment. When a conflict between the two observers raised, the average of these two scores was taken after rounding to the least whole number. The results of the most recent serum ferritin level test of each patient were obtained from their medical records. If the test was older than six months, the patient was not considered for enrollment.

This study was approved by ethical committee of the Faculty of Dental Medicine in Damascus University, Damascus, Syria (resolution no. 570, 28/06/2016).

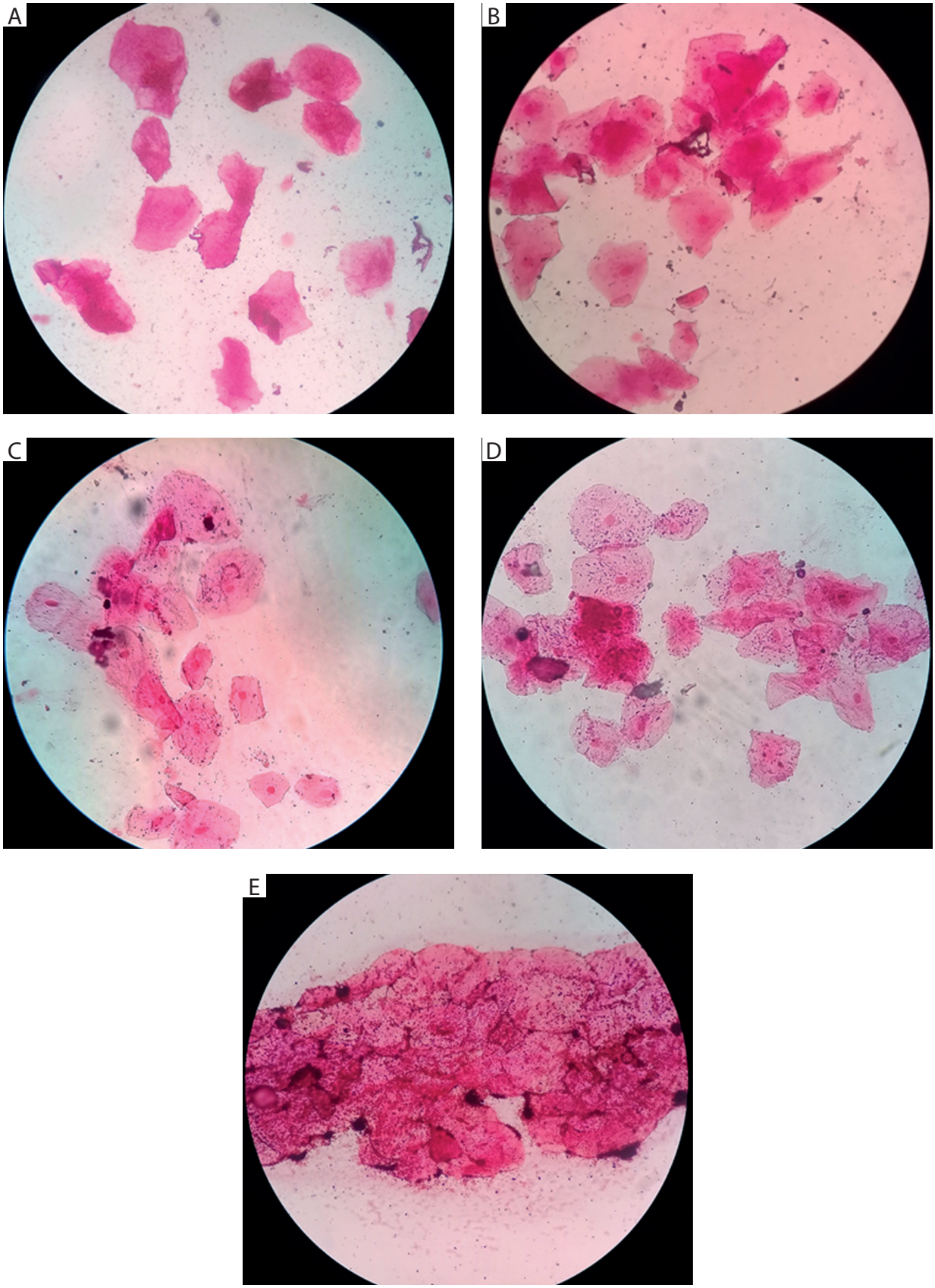

FIGURE 1. Mucosal smear showing the results of Perl's-Prussian blue reaction (× 400 magnification). **A)** Negative reaction, expression score = 0. **B)** Positive reaction, expression score = 1. **C)** Positive reaction, expression score = 2. **D)** Positive reaction, expression score = 3. **E)** Positive reaction, expression score = 4

TABLE 1. Kolmogorov-Smirnov test

	Statistic	df	Significance
Serum ferritin level	0.174	69	0.000

TABLE 2. Correlation of Perls' Prussian positivity with serum ferritin level

Perls' Prussian blue reaction	Number of cases	Average serum ferritin level	*p*-value
Positive	59	2962.19	0.008
Negative	10	1100.40	

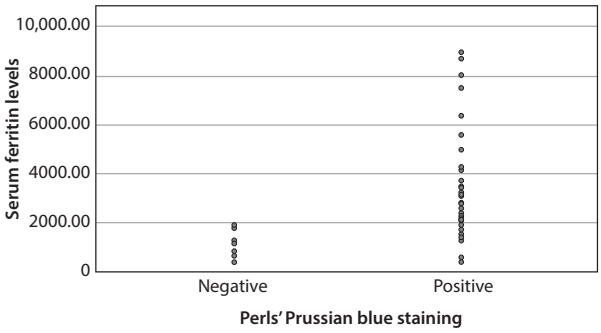

FIGURE 2. Simple scatter of serum ferritin level by Perls' Prussian blue staining

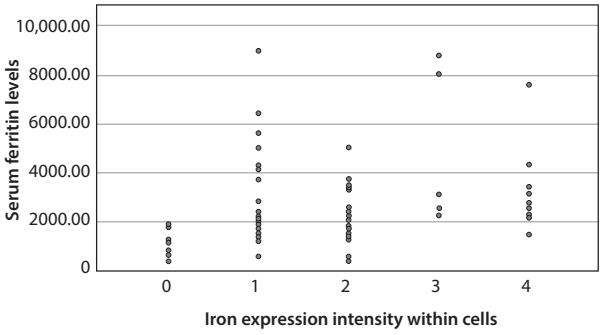

FIGURE 3. Simple scatter of serum ferritin level by iron expression intensity within cells

Statistical analysis was carried out using IBM SPSS Statistics software, v. 23.0 (IBM Corp., Armonk, USA) for Windows. The data was analyzed using Spearman rank's correlation test. Correlations were considered significant at $p < 0.05$.

RESULTS

Fifty-nine out of 69 cases (85.5%) were positive for Perls' Prussian blue reaction. The average ferritin serum level was 2721 µg/l (range, 616-9000 µg/l). Serum ferritin levels were not normally distributed, as assessed by Kolmogorov-Smirnov's test ($p > 0.05$) (Table 1).

Spearman rank's correlation test was used between Perls' Prussian blue staining and serum ferritin level, since Perls' Prussian blue staining is a dichotomous variable and serum ferritin level is a continuous variable with non-normal distribution. There was a moderate positive correlation between Perls' Prussian blue staining and serum ferritin level ($p = 0.008$, $R = 0.315$) (Table 2, Figure 2).

When assessing the expression of cells containing iron granules, a score of 0 to 4 was given based on the estimated amount of iron within the cells. Correlation of iron expression within the cells with serum ferritin level was carried out using the Spearman rank's correlation test, since iron expression within cells is an ordinal variable and serum ferritin level is continuous variable with non-normal distribution. There was a moderate positive correlation between iron expression within the cells and serum ferritin level ($p = 0.012$, $R = 0.301$) (Figure 3).

DISCUSSION

As seen in β-thalassemia major patients, chronic iron overload can result from chronic ineffective erythropoiesis and from multiple blood transfusions. Iron toxicity occurs when iron overload causes non-transferrin bound iron (NTBI) to accumulate in tissues as free iron, leading to organ dysfunction and damage [15].

There are multiple methods of assessing iron level within the body. Some of these methods like serum ferritin level are inaccurate, while more accurate methods like liver biopsy can be invasive, especially when performed repeatedly. Additionally, liver biopsy results are usually poor indicators of iron load in other organs such as cardiac iron load [16]. Therefore, the use of two or more indices of iron status will usually be needed to define the amount of iron and its distribution to different organs.

In this study, exfoliated cells from the buccal mucosa were obtained from 69 β-thalassemia major patients, undergoing a minimum of 15 transfusions. The obtained smears were stained with Perls' Prussian blue stain, and 59 out of 69 cases (85.5%) were positive for Perls' Prussian blue reaction. There was a statistically moderate positive correlation between Perls' Prussian blue reaction positivity and ferritin serum levels.

Our findings were in accordance with those of Rathore *et al.* [17] (82.9% positivity among 35 patients), Chittamestty *et al.* [14] (72.5% positivity among 40 patients), Nandaprasad *et al.* [18] (65% among 100 patients), Baht *et al.* [19] (71.7% among 60 patients), and Gupta *et al.* [20] (61.6% positivity among 60 patients). Of these previous studies, only Baht *et al.* [19] reported a moderate statistical correlation between Perls' Prussian blue reaction positivity and ferritin serum levels. The rest reported a strong statistical correlation between Perls' Prussian blue reaction positivity and ferritin serum levels [14, 17, 18, 20].

These minor changes in the positivity of Perls' Prussian blue reaction and its correlation with ferritin level

might be attributed to differences in sample sizes in each study as well as a discrepancy in commitment of iron chelation therapy between different countries.

In the present study, a moderate positive correlation between iron expression within the cells and serum ferritin level was found. This indicates that the positive staining in exfoliated buccal cells can be used for diagnosing changes in serum ferritin level and thus, suggesting iron overload in the body tissues.

Leekha *et al.* [21] performed a similar study on 40 patients. However, a different method of estimating iron expression was used, with the help of computer-assisted morphometric analysis of exfoliative cells. A positive correlation between iron expression within the cells and serum ferritin level was observed, which is comparable with our results.

The correlation between serum ferritin level and the amount of iron within mucosal cells can be explained by the method, in which the body metabolizes iron and stores it using ferritin or hemosiderin. Chronic ineffective erythropoiesis and repeated blood transfusions lead to uncontrolled iron absorption and efflux into the bloodstream at a rate up to 8-10 mg/day, which gradually causes oversaturation of transferrin and accumulation of non-transferrin-bound iron (NTBI). Unshielded NTBI, which is redox-active and toxic, is eventually taken up by tissue parenchymal cells, especially in the liver, pancreas, and heart [22].

From our results, we observed that the presence of iron granules, as demonstrated by Perls' Prussian blue stain in exfoliative cells of the buccal mucosa, can be reliably used as qualitative and quantitative methods to diagnose an overload of iron in body tissues. However, due to logistical and financial difficulties, a serum ferritin level test could not be performed in the same day the mucosal swap was taken. Therefore, we had to rely on the latest test results available in the patients' records, which might reduce the accuracy of study outcomes.

CONCLUSIONS

Within the limitations of this study, oral exfoliative cytology can be used as a qualitative screening and a diagnostic tool in β-thalassemia patients who undergo repeated blood transfusion, and has potentials as a quantitative diagnostic tool. More studies are needed in order to establish this non-invasive procedure as a reliable screening and diagnostic tool in β-thalassemia patients. We recommend studies that correlate the Perls' Prussian blue reaction with serum ferritin level, and other techniques such as magnetic resonance images and liver biopsies.

CONFLICT OF INTEREST

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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