Cytomorphometric Analysis of Buccal Exfoliated Cells in Patients With Iron Deficiency Anemia

Mahdieh Rajabi-Moghaddam[1], Hamid Abbaszadeh[2], Maryam Mosaffa[3], Hemmat Gholinia[4], Shima Nafarzadeh[5]

[1] *Department of Pathology, School of Medicine, Birjand University of Medical Sciences, Birjand, Iran*

[2] *Department of Oral and Maxillofacial Pathology, School of Dentistry, Birjand University of Medical Sciences, Birjand, Iran*

[3] *Student Research Committee, Babol University of Medical Sciences, Babol, Iran*

[4] *Institute of Health, Babol University of Medical Sciences, Babol, Iran*

[5] *Department of Oral and Maxillofacial Pathology, School of Dentistry, Babol University of Medical Sciences, Babol, Iran*

Received: 29 Feb. 2020; Accepted: 28 Jul. 2020

Abstract- Iron deficiency anemia can cause histopathologic alterations in the oral mucosa. Exfoliative cytology is a cheaper and less aggressive method for early diagnosis. The aim of this study was to compare the cytological and cytomorphometric characteristics of buccal exfoliated cells of iron deficiency anemic patients with those of healthy controls. This case-control study compared a group of 40 patients with iron deficiency anemia (IDA) with an age- and gender-matched control group (C) of 40 healthy individuals. The buccal mucosal smears were stained using the Papanicolaou technique for cytological analyses. Cellular clumping, cytoplasmic diameter (CD), nuclear diameter (ND), cytoplasmic area (CA), nuclear area (NA), nucleus to cytoplasmic area ratio (NA/CA), cellular and nuclear pleomorphism, micronuclei (Mn), binucleation, bacterial colonies, and keratin flakes were evaluated using a light microscope and digital image analysis. Mean values for IDA and C groups were: bacterial colonies (1.88 and 0.65; *P*=0.002); CA (2209.88 and 1687.79 μm²; *P*=0.006); Mn (1.60 and 0.60; *P*=0.02). Significant increases in bacterial colonies, CA and Mn were seen for the IDA group. The number of cellular clumps, CD, ND, NA, NA/CA, cellular and nuclear pleomorphism, binucleation, and the number of keratin flakes didn't show significant differences between studied groups (*P*>0.05). There wasn't any significant difference with respect to overall atypia. This study revealed that IDA was able to induce significant changes in CA and Mn of the oral epithelial cells. Exfoliative cytology and cytomorphometry can be used as a tool to assess the mucosal changes in IDA patients.

Acta Med Iran 2020;58(9):424-429.

Keywords: Cytology; Iron deficiency anemia; Iran

Introduction

Anemia defines as an abnormal decrease in the number of circulating red blood cells, a decrease in the amount of hemoglobin, and a decrease in the volume of red cells in the blood unit. Iron deficiency is the most common nutritional disorder worldwide, and iron deficiency anemia is still the most common cause of anemia throughout the world (1).

Oral mucosa is involved in certain types of systemic disorders such as anemia, leukemia, vitamin deficiency, and many of infectious diseases. Oral manifestations of iron deficiency anemia include angular cheilitis, atrophic glossitis, and generalized atrophy of oral mucosa (2).

Of course, all cases of iron deficiency anemia don't lead to clinically detectable oral lesions, and it is unclear whether microscopic changes occur in these cases of iron deficiency anemia that do not have an obvious mucosal abnormality or not (2,3).

There are several methods for assessing the oral mucosa, including tissue biopsy. In most instances, the biopsy is not usually applicable due to aggressiveness and patient rejection, especially in the absence of clinically visible changes. It seems that the use of exfoliative cytology with lower cost, less aggressiveness, and lack of injury to oral tissues is one of the best methods for evaluation of oral mucosa, especially for early detection of mucosal changes (4).

Corresponding Author: H. Abbaszadeh
Department of Oral and Maxillofacial Pathology, School of Dentistry, Birjand University of Medical Sciences, Birjand, Iran
Tel: +98 9158002580, Fax: +98 5632381700, E-mail address: hamidabbaszade@yahoo.com

There are little and conflicting results (1,5,6) about cytological changes of oral mucosa in iron deficiency anemia patients; such a study has not been reported in the Iranian population. So, the aim of this study was to compare the cytological and cytomorphometric characteristics of the buccal exfoliated cells of iron deficiency anemic patients with those of healthy controls in an Iranian population.

Materials and Methods

This case-control study compared a group of 40 patients with iron deficiency anemia (IDA) with an age- and gender-matched control group (C) of 40 healthy individuals. Because of the prevalence of iron deficiency anemia in females, females were selected for two studied groups, and the age range of participants was 15-40-year-old.

A hematologist confirmed the diagnosis of iron deficiency anemia. The criterion for anemia was hemoglobin less than 120 g/L in females. Criteria for iron deficiency were iron plasma level below than 30 µg/dl, ferritin plasma level below than 15 µg/land TIBC (total iron-binding capacity) more than 360 µg/dl. Abnormality of the two above mentioned parameters was considered as iron deficiency anemia (7).

Exclusion criteria were as follows: systemic diseases such as leukemia, lymphoma, rheumatismal diseases, diabetes mellitus, and megaloblastic anemia; the history of treatment such as radiation therapy of head and neck area and taking immunosuppressive or cytotoxic drugs; localized pathologic changes such as macroscopic abnormalities, gingival and periodontal diseases; individuals with harmful habits like smoking and alcohol consumption; individuals who are affected to infectious or inflammatory diseases (such as inflammatory bowel disease); pregnant women; persons with removable dentures (1,2).

The study was approved by the ethics committee (Code: REC.1392.155). Patients with a clinical diagnosis of iron deficiency anemia who referred to an internist and healthy individual who met the exclusion criteria were included in the study for additional blood evaluation. Initially, informed consent was obtained from volunteers who participated in our study. A blood sample was being taken. Complete blood count or CBC test (containing hemoglobin, hematocrit, platelet count, red blood cell count, mean corpuscular volume or MCV, Mean corpuscular hemoglobin or MCH, Mean corpuscular hemoglobin concentration or MCHC, Red Cell Distribution Width or RDW), peripheral blood smear, serum iron, serum ferritin, and TIBC were assessed. Patients who met the inclusion criteria for iron deficiency anemia were considered as an iron deficiency group; healthy individuals with normal blood test results were considered as a control group.

In the next step, oral exfoliative cytological smears were prepared from buccal mucosa (1). The participants were asked to rinse their mouth with water. Then, smears were prepared with the exfoliative cytology method using cytobrush (Papette®, Wallach Surgical Devices, USA). The cytobrush was placed in contact with oral epithelium in the area. Using constant medium pressure, the brush was spun 10-17 times. The pressure of the hand during the preparation of the smear was such that only the superficial epithelial cells of the mucosa were harvested, and no bleeding occurred. The heads of cytobrush were immersed in preservative solution (PreservCyt®, Fisher, USA) and transferred to the laboratory for smear preparation. Using the cytosine technique, suspensions containing exfoliated cells were centrifuged for 10 minutes in 2500 revolutions per minute (rpm), and exfoliated cells were transferred on glass slides (8).

The cytological smears stained with the Papanicolaou technique (PAP stain) and evaluated under a light microscope at 400X magnification. The slides were stained within a maximum of three days of sampling according to the standard Papanicolaou staining method as follows:

The slides were fixed in 95% ethanol for 15 minutes, followed by immediate dipping in 50% ethanol for 2 minutes. After that, the slides were washed in tap water for 10 seconds. After the water had been removed from the slides using tissue papers, the slides were kept in Harris hematoxylin stain for 1 minute. Then the slides were washed in tap water until being cleared. 0.5% acid alcohol was used as a 2-3 quick dips. The nuclear stain was checked under the light microscope to ensure the clarity of the nuclei. The slides were washed in water for ten dips, followed by ten dips in two changes of 95% ethanol. Immediately, the slides were placed in O-G-6 for 3 minutes. The slides were dipped in two changes of 95% ethanol for ten dips each. After that, the slides were placed in EA-50 for 4 minutes. The slides were dipped in three changes of 95% ethanol for ten dips each. Then, the slides were dipped in three changes of absolute ethanol for ten dips each. The slides were dipped in three changes of xylene for 15 dips each. Finally, the slides were mounted in DPX (6).

For qualitative cytologic evaluation, specimens were assessed under a light microscope (Olympus BX41,

Olympus Corporation, Tokyo, Japan) at 400X magnification. For cytomorphometric analysis, images were captured from cytological smears by the camera (Olympus Corporation, Tokyo, Japan) attached to the light microscope, and calculations were done in the captured image using Analysis LS Starter software, version 5.1 (Olympus Soft Imaging Solutions, Olympus Corporation, Tokyo, Japan).

The buccal mucosal smears were stained using the Papanicolaou technique for cytological analyses. Cellular clumping, cytoplasmic diameter (CD), nuclear diameter (ND), cytoplasmic area (CA), nuclear area (NA), nucleus to cytoplasmic area ratio (NA/CA), cellular and nuclear pleomorphism, micronuclei (Mn), binucleation, bacterial colonies, and keratin flakes were evaluated using a light microscope and digital image analysis.

In each sample, assessment of cytological features was as follow structural features including cellular clumping and nucleus to cytoplasmic area ratio (NA/CA); nuclear features including nuclear diameter (ND), nuclear area (NA), nuclear pleomorphism, and binucleation; cellular features including cytoplasmic diameter (CD) [greatest diameter], cytoplasmic area (CA), cellular pleomorphism and micronuclei; other features including the presence of candida, number of bacterial colonies, and number of keratin flakes (anuclear amorphous eosinophilic plate-like material) (6).

We assessed the above-mentioned cytological features in the first 100 cells (in a background devoid of bleeding, necrosis, or exudate) (6). The selected cells should have proper staining and defined cytoplasmic borders; if we observed overlapping of cells and indistinct cellular membranes, those cells were not included in the study (6).

Also, we evaluated the overall atypia in the studied groups. The presence of two or more abnormal cytologic features, including nuclear pleomorphism, cellular pleomorphism, binucleation, micronuclei, and abnormal NA/CA, were indicative of the presence of atypia in that specimen (6). For differentiation between reactive atypia (presence of immature cells formed in the process of healing or regrowth of the squamous epithelium that often follows the treatment of dysplasia and other conditions such as infections) from atypia observed in premalignant squamous epithelium, we considered reactive atypia as aggregates of immature keratinocytes often with increased nuclear to cytoplasmic ratios and sometimes prominent nucleoli, but with no other nuclear abnormalities; samples with reactive atypia were excluded from our study (6).

Criteria for identifying micronucleus

1. Diameter less than $\frac{1}{3}$ the main nucleus.
2. Non-refractivity (to exclude small stain particles).
3. Color the same as or lighter than the nucleus (to exclude large stain particles).
4. Location within 3 or 4 nuclear diameters of a nucleus and not touching the nucleus (to make frequency measurements meaningful).
5. No more than two micronuclei are associated with one nucleus (6).

Cellular and nuclear pleomorphism assay

At first, images were captured from cytological smears using the camera attached to the microscope; Cytoplasmic areas of 100 cells and their nuclear area were calculated in the captured image using Analysis LS Starter software (Olympus Corporation, Tokyo, Japan). Then it was determined whether the distribution of data (CA and NA) was normal or not using Kolmogorov-Smirnov statistical test. If $P<0.05$, it meant that the distribution of data was not normal, and there was cellular or nuclear pleomorphism; conversely, if $P>0.05$, it meant the distribution of data was normal, and there was no cellular or nuclear pleomorphism.

Nucleus to cytoplasmic area ratio (NA/CA) assay

After calculating the mean nuclear area (NA) and cytoplasmic area (CA) in each sample, the nucleus to cytoplasmic area ratio (NA/CA ratio) was obtained by this fraction: mean nuclear area/ mean cytoplasmic area. NA/CA ratio was considered abnormal if the NA/CA ratio$>\frac{1}{3}$ (6).

To compare studied groups, the obtained data entered into SPSS version 20 software and analyzed by statistical tests (Kolmogorov-Smirnov, T-test, and Chi-Square). $P<0.05$ was considered significant.

Results

The mean ages of anemic and healthy individuals were 34.7±6.26 and 33.83±6.93-year-old, respectively.

Table 1 shows comparisons of quantitative cytological features (cytomorphometric characteristics) in studied groups. The number of cellular clumping, the number of keratin flakes, the number of binucleation, cytoplasmic diameter (CD), nuclear diameter (ND), nuclear area (NA), and NA/CA ratio didn't show any significant differences between iron deficiency anemia group and control group($P>0.05$) (Table1).

Table 1. Comparison of quantitative cytological features between patients with iron deficiency anemia and healthy individuals

Feature	Group	N	Mean	Standard Deviation	*P*
bacterial colonies	Iron deficiency anemia	40	1.88	2.15	0.002*
	healthy	40	0.65	1.07	
keratin flakes	Iron deficiency anemia	40	0.8	0.79	0.19*
	healthy	40	0.57	0.74	
Cellular clumping	Iron deficiency anemia	40	3.18	1.78	0.62*
	healthy	40	3.59	4.69	
Micronuclei	Iron deficiency anemia	40	1.60	2.46	0.02*
	healthy	40	0.60	1.21	
Micronucleated cells	Iron deficiency anemia	40	1.15	1.71	0.02*
	healthy	40	0.43	0.84	
binucleation	Iron deficiency anemia	40	0.13	0.46	0.25*
	healthy	40	0.28	0.67	
Cytoplasmic diameter (µ)	Iron deficiency anemia	40	60.47	7.13	0.74*
	healthy	40	61.07	9.01	
Nuclear diameter(µ)	Iron deficiency anemia	40	8.73	1.54	0.54*
	healthy	40	8.94	1.49	
Nuclear area (μ^2)	Iron deficiency anemia	40	156.54	64.78	0.22**
	healthy	40	140.41	53.70	
Cellular area (μ^2)	Iron deficiency anemia	40	2209.88	1028.95	0.006**
	healthy	40	1687.79	531.84	
Nuclear to cytoplasmic ratio	Iron deficiency anemia	40	0.09	0.08	0.20*
	healthy	40	0.07	0.03	

* *t*-test; ** Mann-Whitney test

Mean values of the number of bacterial colonies and cytoplasmic area for iron deficiency group and control groups were: bacterial colonies (1.88±2.15 and 0.65±1.07); cytoplasmic area (2209.88±1028.95 µm²and 1687.79±531.84 µm²); Mean numbers of bacterial colonies and mean cytoplasmic areas were higher in iron deficiency group. There were significant differences in the mean number of bacterial colonies and mean cytoplasmic areas between studied groups (P=0.002<0.05 and P=0.006<0.05, respectively) (Figure 1).

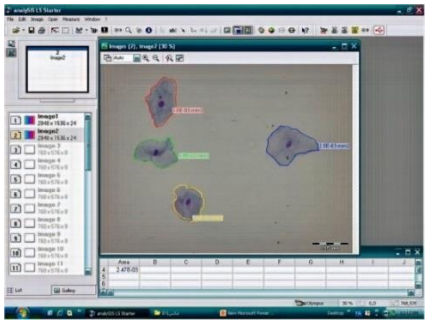

Figure 1. Measurement of the cellular area in a captured image by Analysis LS Starter software

Mean values of micronucleus and micronucleated cells (cells with micronucleus) for iron deficiency group and control groups were: micronucleus (1.60±2.46 and 0.60±1.21); micronucleated cells (1.15±1.71and 0.43±0.84). The number of micronuclei and micronucleated cells (cells with micronucleus) was higher in the iron-deficiency anemia group than the control group. There were significant differences in the mean number of micronuclei (Mn) and micronucleated cells between studied groups (P=0.02<0.05) (Figure 2).

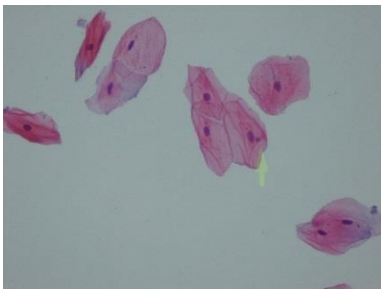

Figure 2. Micronuclei in smears of patients with iron deficiency anemia (400X magnification)

Table 2 shows comparisons of qualitative cytological

features in studied groups. There weren't any significant differences between studied groups with respect to the presence or absence of candida, cellular pleomorphism, nuclear pleomorphism, and overall atypia (P>0.05) (Table 2).

Table 2. Comparison of qualitative cytological features between patients with iron deficiency anemia and healthy individuals

Group / Feature		N (%)		*P*
		Iron deficiency anemia	healthy	
Candida	**presence**	13 (32.5%)	16 (40%)	0.45*
	absence	27 (67.5%)	24 (60%)	
Cellular pleomorphism	**presence**	6 (15%)	5 (12.5%)	0.74*
	absence	34 (85%)	35 (87.5%)	
Nuclear pleomorphism	**presence**	4 (10%)	2 (5%)	0.39*
	absence	36 (90%)	38 (95%)	
Atypia	**presence**	7 (17.5%)	5 (12.5%)	0.53*
	absence	33 (82.5%)	35 (87.5%)	

**Chi*-Square Test

Discussion

Cellular alterations of oral mucosa occur early without visible clinical signs and symptoms. Exfoliative cytology is the most convenient method of collection of the oral epithelial cells. So, the aim of the present study was to compare the cytological and cytomorphometric characteristics of buccal exfoliated cells of iron deficiency anemic patients with those of healthy controls.

There are little and conflicting results about cytological changes in oral mucosa in iron deficiency anemia patients. In the study of Macleod RI *et al*., on oral exfoliative cytology, no morphologic and morphometric changes were seen in iron deficiency anemia and non-vitamin B12 megaloblastic anemia (5). In Gururaj N *et al*., the study on cytological changes in the buccal mucosal cells of iron deficiency anemia, there was an increase in the cytoplasmic diameter, nuclear diameter, and nuclear to cytoplasmic ratio of iron deficiency anemia patients when compared to the normal values. In Sumanthi J *et al*., study, a significant increase was seen in the average nuclear diameter and nuclear to cytoplasmic ratio of the iron-deficiency anemia group when compared to the control group, but the average cytoplasmic diameter did not show any statistical difference among the two groups (1).

In our study, the number of micronuclei and micronucleated cells was significantly higher in the iron-deficiency anemia group than the control group. The frequency of occurrence of micronuclei is a measure of chromosome breakage in early cell divisions. The number of micronuclei is known to increase with carcinogenic stimuli. Micronuclei can be identified in cells like lymphocyte, erythrocyte, and exfoliated epithelial cells. The study of micronuclei in exfoliated cells is a reliable tool for studying the risk of malignancy (4). Iron deficiency, especially the severe chronic forms, is associated with an elevated risk for squamous cell carcinoma of the esophagus, oropharynx, and posterior mouth (3). So, it may be possible that iron deficiency anemia in mild and moderate forms can induce cytologic alterations such as an increase in the number of micronuclei, which can be an early event in the process of malignant changes. Therefore, assessment of micronuclei and micronucleated cells in buccal exfoliated cells of iron deficiency anemic patients is recommended for evaluating atypical changes of the oral mucosa.

In our study, the number of bacterial colonies was significantly higher in iron deficiency anemia patients compared to the control group. Patients with iron-deficiency anemia may complain of fatigue, easy tiring, and lack of energy. So, the above-mentioned finding can be justified according to poor oral hygiene in these patients due to impatience in brushing and maintaining proper oral hygiene.

In our study, no significant differences were seen in cytoplasmic diameter, nuclear diameter, nuclear area, and NA/CA ratio between the iron-deficiency anemia group and the control group. These findings are consistent with Macleod RI *et al*., study (5) but are inconsistent with Gururaj N *et al*., study (6). A significant difference in the cytoplasmic area between studied groups in our study is inconsistent with Macleod RI *et al*., study (5). With respect to cytoplasmic

diameter, there is consistency between our study and Sumanthi J *et al*., study (1), but with respect to nuclear diameter and nuclear to cytoplasmic ratio, there is a contradiction between our results and Sumanthi J *et al*., results (1). The reason for these differences and inconsistencies may be related to the lack of attention to confounding variables in these studies, such as duration and severity of iron deficiency anemia. It is recommended that in the future research on cytologic alteration of iron deficiency anemia, patients of different severity of iron deficiency anemia (according to hematological findings) be evaluated and compared with control groups. Also, the duration of iron deficiency anemia is an unknown variable. It may be dependent on the culture of people (when will they seek treatment for their symptoms of anemia) and the quality of health care delivery in different countries (early detection of diseases in health systems around the world). So, it is recommended that cytologic alteration of the oral mucosa of iron deficiency anemia is evaluated in different countries and cultures.

Paraizo JU *et al*., observed a significant increase in the nuclear area for sickle cell anemia compared to the control group, but no other morphological differences were found between the groups (9).

This study revealed that iron deficiency anemia is able to induce significant cytologic oral mucosal changes. Exfoliative cytology and cytomorphometry can be used as a tool to assess the mucosal changes in iron deficiency anemia patients.

Acknowledgments

We thank the research vice-chancellor of our university for their support of this study.

References

1. Sumanthi J, Reddy GS, Anuradha C, Sekhar PC, Prasad LK, Reddy BR. A study on cytomorphometric analysis of exfoliative buccal cells in iron deficiency anemic patients. Contemp Clin Dent 2012; 3:S156-9.
2. Scott J, Valentine J, St Hill C, West C. Morphometric analysis of atrophic changes in human lingual epithelium in iron deficiency anaemia. J Clin Pathol 1985; 38:1025-9.
3. Neville BW, Daam DD, Allen CM, Chi AC. Oral and maxillofacial pathology.4th ed. USA: Missouri: Saunders co, 2016:377,773.
4. Kohli M, Ahuja P, Mehendiratta M, Sharma M, Dutta J. Micronucleus Assay: An early diagnostic tool to assess genotoxic changes in patients with tobacco use, oral leukoplakia and oral submucous fibrosis. J Clinic Diagn Res 2017; 11: ZC28.
5. Macleod RI, Hamilton PJ, Soames JV. Quantitative exfoliative oral cytology in iron-deficiency and megaloblastic anemia. Anal Quant Cytol Histol 1988; 10:176-80.
6. Gururaj N, Sivapathasundharam B, Sumathy N. Cytological findings in iron deficiency anemia. Indian J Dent Res 2004; 15:126-8.
7. World Health Organization. Iron deficiency anaemia: assessment, prevention and control: a guide for programme managers. 1st ed. Geneva: WHO, 2001.
8. Sharbatdaran M, Abbaszadeh H, Siadati S, Ranaee M, Hajian-Tilaki K, Rajabi-Moghaddam M. Assessment of oral cytological features in smokers and nonsmokers after application of toluidine blue. Diagn Cytopathol 2017; 45:513-9.
9. Paraizo J, Rech I, Azevedo-Alanis L, Pianovski M, De Lima A, Machado M. Cytomorphometric and cytomorphologic analysis of oral mucosa in children with sickle cell anemia. J Cytol 2013; 30:104-8.