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The Effect of Medications in Buccal Epithelial Cells of Behcet's Disease Patients (Cytomorphometrical Study)

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Abstract: ***Background: Behcet's disease (BD) is a multi- systemicchronic vacuities passing with alternating stages of flare up and remission. The disease is characterized by repetitive aphthous ulcers in addition to either of the following: genital ulcers, articular, cutaneous as well as ocular symptoms and central nervous system lesions. Oral Exfoliative cytology is considered as important technique for understanding many changes (normal, abnormal) happened on cells. Aim: the aim of current study is to determine the alterations in cyto-morphometry of buccal exfoliated specimens inBehcet's disease patients caused by steroid or cholchicine treatment. Material and methods: Supra-basal cells specimens were obtained from apparently normal (away from aphthus lesions) buccal mucosa ofBehcet's diseasepatients under treatment (steroid, cholchicine) and newly diagnosed untreated cases in addition to healthy subjects. The "Papanicolaou method" has been used for staining histologic specimens. Cytomorphometric evaluation was done using light microscope and image analysis system. Results: The untreatedBehcet's disease group was associated with significant reduction in cytoplasmic and nuclear area, but preserving the ratio between the two cellular characteristics compared to healthy group.Both steroids and colchicine treatments were associated with a statistically significant increase in cytoplasmic area compared to untreated cases. However, the nuclear area showed a noticeable reduction inBehcet's diseasetreated with steroids resulting in a comparable reduction in nuclear to cytoplasmic area ratio in treated cases compared to untreated. Conclusion: the study demonstrated thatBehcet's disease process and treatment can reflect at the cellular level. Such micro-level changes my give a key to future understanding of disease and treatment pathological processes.***

Keywords: Behcet's disease, cytomorphometric analysis, cytoplasmic area, nuclear area. Steroid, colchicines

1. Introduction

Being an inflammatory disorder, Behcet's disease(BD) is characterized by recurrent mucocutaneous lesions (oral, genital, and skin). Itwas first diagnosed by Hulusi Behcet, a Turkish dermatologist, in 1937 when treatingthree patients with aphthous ulcers, genital ulcers and eye lesions [1]. The effect of Behcet's disease affects all the body organs [2,3]. Turkey is one of the "ancient Silk Road" countries in which BD is a known illness[4].

The etio-pathogenesis and clinical features of BD have been discussed in manystudies[5].(Oral and genital ulcers, uveitis, and skin lesions are essential symptoms which gives a clue to the diagnosis of Behcet's disease. Anti-inflammatory agents like systemic and topical steroid are commonly-used treatments of Behcet's disease. The use of such agents for a long time could have serious side effects, requiringa simultaneous use of other immune-suppressive medications, such as thalidomide, interferon alpha, azathioprine, and colchicine[1,5]. The effect of immune-supressive medications on the oral mucosa cells was documented by researchers [6,7,8] .These cellular effects could be dose-related, with carcinomas of gingival cellsbeing frequently reported as an end result[9].After careful search of online medical repositories and databases (Pubmed, Google Scholar, Ovid) using the combination of the following terms: Behcet's disease, Steroids and cytomorphometry) no published article was found about the effect of steroid treatment on oral mucosal cells of Behcet's disease.

Exfoliative cytology is defined as an easy way for the morphologic and morphometric evaluation of exfoliated cells from buccal mucos[10]. It is also a cheap and simple procedure for diagnosing lesions in oral mucosal specimens. In addition, cytomorphometric changes can be determinedat the early stages [11].

In the assessment of exfoliative cytology, quantitative techniques are considered more objective and precise as they depend upon the evaluation of quantitative values such as alterations in the cytoplasm and nucleus, noting that the changes in nucleus/cytoplasm (N/C) ratio may consolidate many disease in terms of diagnostic sensitivity [7].This technique has been made into effect for evaluating certain structural changes of cells, whether in normal tissues or abnormal ones[12].Moreover, a lot of studies have quantified certaincytoplasmic and nuclear variables as possible histologic predictors of biologic pathway for criteria of the diagnosis of aggressive changes to be established[13]. Currently, with the recent progression of "image analysis software", the application of quantitative methods altogether has allowed the potential precision of studies regarding cytomorphometryto be improved [14].

The aim of present study is to evaluate the clinical features of BD cases and investigate quantitative cytologic alterations of oral mucosal cells gathered from BD patients who were taking medications (steroid, colchicine)and new cases(prior to inception of treatment).

2. Materials and Methods

This study included 75 BD patients(52 males and 23 females) were divided into three equally sized groups (untreated, steroid treated and colchicine treated) and 25 apparently healthy subjects(17 males and 8 females).The age of BD patients and controls ranged from 16 to 60 years. The study group characteristics are found in Table (1). Smears of buccal mucosal cells were obtained from Behcet's disease patients recruited in dermatology department of Baghdad teaching hospital in the medical city. A written consent was obtained from each study participant. The socio-demographic and past medical history was obtained from each subject using a structured questionnaire format in a private face to face interview session.

Strict inclusion criteria were applied to Behcet's disease patients. Excluding those with radiotherapy, alcohol consumption, diabetes mellitus, anemia, or smokers. The final study groups used were: BD cases on colchicine treat men, BD cases on steroid treatment, new cases with BD (without treatment) and healthy subjects as "control group". The whole specimens were obtained from normal buccal mucosal cells (being as far as possible from nearby aphthus lesions.

2.1 Preparation of specimen

The subjects have been instructed to rinse their mouth with water, then a gauze swab was used to dry the buccal mucosa in order for surface debris and excess saliva to be removed. A disposable pap smear brush was used to collect smears from non-aphthous areas of the mouth (in case of BD patients) and move them to glass slides. Similarly, smears from controls can be collected in a similar way. The slides were then directly fixed with95% ethyl alcohol and stained afterwards following the "papanicolaou technique"[15].

2.2Assessment of Cyto-morphometry

Unfolded with clear outlines, one hundred cells per subject have been identified by moving the slide from the left-to-right in a zigzag order. The cells used for such smear analysis should be consistent, supra-basal, unclamped, and mono-layered. With the use of a microscope equipped with a 20X, cells can be seen as images to be transmitted to a video camera Eyepiece for TV 0.3 M Pixel VCE-PW1 displayed on a video monitor (China). For the purpose of image analysis for nuclear area (NAr), cytoplasmic area (CAr), and nucleo cytoplasmic ratio(NAr/CAr) figure 1,2using "motic image" version 0.3(x86) computer software, a screen shot of each slide was captured, saved, and transferred to the computer.

2.3 Statistical Analysis

IBMSPSS computer software version 23 in combination with Microsoft Excel were used for statistical analyses. Semirnov-kolmogorov test showed that the outcome quantitative variables (NAr,CAr, and NAr/CAr)significantly departed from normal distribution. The median, inter–quarter range and the mean rank were therefore used as measures of central tendency and the non-parametric Kruskall-Wallis test was used to assess the statistical significance of difference between study groups. In addition, the Mann Whitney test was used for comparisons of two groups.

3. Results

The range of BD patients age was 16-60 years with amean age of 34.9 years, while healthy controls ranged between 18-45 years with a mean age 28.1 years.

Table 1 showed that the three BD groups had a significantly smaller median area of cytoplasm with 4980.7 um^2,6311.2 um^2 and 7529 um^2respectively in BD without treatment, colchicine treated, and steroid treated groups when compared with 11530.7 um^2area in healthy control subjects. In addition, the median CAr in treated groups (whether steroid or colchicine) was higher than that found in untreated one. This difference being more obvious with steroid treatment.

The median of NAr in the three BD groups (157.6 um^2, 165.1 um^2 and 142.1 um^2 in BD without treatment, colchicine treated, and steroid treated groups respectively) was significantly smaller than healthy subjects(388.5 um^2). The median NAr in BD on colchicine treatment was slightly, but not significantly higher than that of untreated BD cases, while in steroid treated cases the NAr was significantly lower than untreated BD cases.

The NAr/Car ratio was maintained almost constant in untreated BD cases (0.033) and healthy controls (0.032). Treatment with steroid and colchicine among BD subjects is associated with a statistically significant reduction in this ratio (colchicine 0.026 and steroid 0.02) compared to untreated BD group (0.033).

Table 1: (cytomorphometrical analysis of study groups)

	Study group				
	Healthy control	*Newly diagnosed (untreated) BD cases*	*BD cases+steroid treatment*	*BD cases+Colchicine treatment*	*P*
Cytoplasmic area (um2)					<0.001
Range	(9417.9 to 14775.9)	(3440 to 5924.7)	(4260.6 to 9949.8)	(4373 to 8832.6)	
Median	11530.7	4980.7	7529.6	6311.2	
Inter-quartile range	(10146.7 to 12463.1)	(4418.2 to 5289.1)	(6339.7 to 8518)	(6063.3 to 6610.5)	
N	25	25	25	25	
Mean Rank	87.7	15.6	56.2	42.5	
P (Mann-Whitney test) for difference in median between:					
Healthy control x Newly diagnosed (untreated) BD cases<0.001					
Healthy control x BD cases+steroid treatment<0.001					

	Study group				
	Healthy control	*Newly diagnosed (untreated) BD cases*	*BD cases+steroid treatment*	*BD cases+Colchicine treatment*	*P*
Healthy control x BD cases+Colchicine treatment<0.001					
Newly diagnosed (untreated) BD cases x BD cases+steroid treatment<0.001					
Newly diagnosed (untreated) BD cases x BD cases+Colchicine treatment<0.001					
BD cases+steroid treatment x BD cases+Colchicine treatment=0.0016					
Nuclear area (um2)					<0.001
Range	(259.5 to 452.8)	(121 to 197.5)	(104.2 to 237)	(116 to 214.7)	
Median	388.5	157.6	142.1	165.1	
Inter-quartile range	(338.8 to 397.5)	(153.5 to 168.6)	(129.4 to 152.4)	(148.1 to 174.9)	
N	25	25	25	25	
Mean Rank	88	42.8	27.6	43.7	
P (Mann-Whitney test) for difference in median between:					
Healthy control x Newly diagnosed (untreated) BD cases<0.001					
Healthy control x BD cases+steroid treatment<0.001					
Healthy control x BD cases+Colchicine treatment<0.001					
Newly diagnosed (untreated) BD cases x BD cases+steroid treatment=0.0059					
Newly diagnosed (untreated) BD cases x BD cases+Colchicine treatment=0.66[NS]					
BD cases+steroid treatment x BD cases+Colchicine treatment=0.0215					
Nuclear to cytoplasmic area ratio					<0.001
Range	(0.024 to 0.04)	(0.024 to 0.049)	(0.012 to 0.029)	(0.019 to 0.034)	
Median	0.032	0.033	0.02	0.026	
Inter-quartile range	(0.031 to 0.034)	(0.031 to 0.034)	(0.016 to 0.023)	(0.022 to 0.029)	
N	25	25	25	25	
Mean Rank	72.2	72.8	17.5	39.5	
P (Mann-Whitney test) for difference in median between:					
Healthy control x Newly diagnosed (untreated) BD cases=0.92[NS]					
Healthy control x BD cases+steroid treatment<0.001					
Healthy control x BD cases+Colchicine treatment<0.001					
Newly diagnosed (untreated) BD cases x BD cases+steroid treatment<0.001					
Newly diagnosed (untreated) BD cases x BD cases+Colchicine treatment<0.001					
BD cases+steroid treatment x BD cases+Colchicine treatment<0.001					

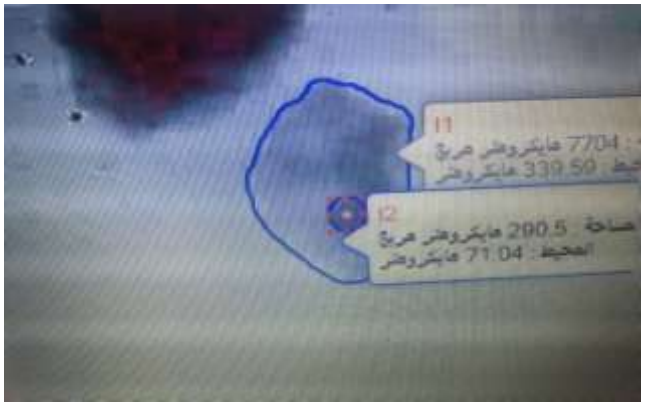

Figure 1: The encirclement of nuclear and cytoplasmic boundaries of bucall cell of BD cases on image taken on digital manner (x20)

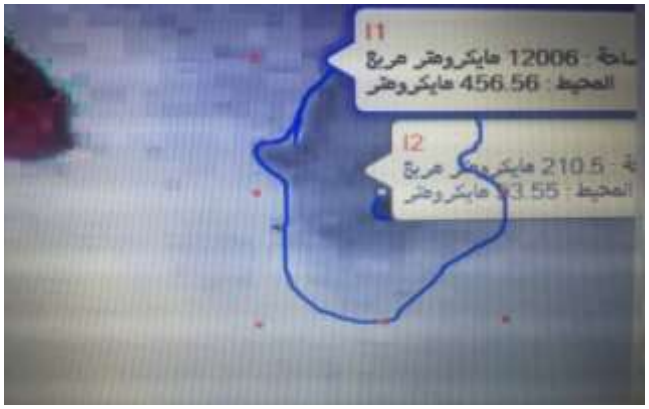

Figure 2: The encirclement of nuclear and cytoplasmic boundaries of bucall cell of control subjects on image taken on digital manner (x20)

4. Discussion

The current study examinedcytomorphometrically the buccal mucosal cells of BD cases (untreated and treated) and healthy volunteers.

The current study showed that BD is associated with cellular downsizing. Both NAr and CAr significantly decreased among untreated patients compared to healthy controls, but

their ratio was nevertheless maintained. This is agree with another study that reported significantly smaller area of cytoplasmand nucleus that happened due to BD affects to be considered as an altered factor for buccal epithelial cells[16].

Another study using dissimilar cytomorphometrical parameter (volume instead of area) three dimensions taken from two dimensional digital image by using specific formula. The finding of this paper also agreed with current study findings, showing a significantly smaller volume of cells and nucleus in new cases Behcet's disease when compared to controls, but the NAr/CAr ratio was also disturbed by the disease process, which is different from the current study findings [8].The reduction in areas of buccal mucosal cells and their nuclei occurred due to elevated level of oxidative stress in Behcet's disease rather than controls [17,18].

The current study showed that colchicine medication in BD patients was associated with a significantly larger Car compared to untreated BD group. It was also associated with a slightly larger nucleus which did not reach the level of statistical significance. In addition, the NAr/CAr ratio which was originally maintained in untreated BD patients despite the presence of the disease was disturbed by the treatment causing a smaller ratio in treated Vs untreated BD. That may be related to mechanism of action of this medication as antimitotic when binds to tubulin and block the cycle of cell[19]One previous study investigated the effect of cholchicine as immunosuppressive medication and its outcome disagree with current study. It revealed that cytoplasmic volume was significantly smaller than untreated (new cases) of BD, in contrast to a significantly larger Nuclear Volume compared resulting in a larger N/C ratio in treated cases. The paper concluded that colchicine treated cases were considered premalignant due to its alteration on cyto-morphometric parameters.[8]. This discrepancy between Kara work and the current study may be related to a combination of factors, which include differences in dose and duration of treatment and disease severity in addition to small sample size and use different cyto-morphometric parameter (volume).

The current study was the first to evaluate the effect of steroid medication on BD using exfolative cytology technique. It showed that the CAr increased with steroid treatment compared to new untreated cases, but not enough to counteract the shrinkage imposed by untreated BD compared to healthy controls. In addition, the NArwas significantly smaller after treatment resulting in a comparable reduction in ratio.

The alterations in CAr and NAr may be due to steroids mode of action. A varied mode of action can be observed with steroids, one of which is the signaling mode of action that affect the phospholipids and channels of ions by receptors on surface of cell. The other one is the classic mode "passive diffusions" altered gen transcriptions [20]. Neuro-active steroids protect cells from oxidative stress, so the decrease in CAr expected in BD which is attributed to oxidative stress can be counteracted resulting in eventual increase in CAr [12].

5. Conclusions

The inflammatory process of BD has far reaching effects on human body. At the cellular level it is associated with shrinkage in cytoplasmic and nuclear size of oral mucosal cells. This reduction in cell size will not alter the cellular proportions, maintaining the nuclear to cytoplasmic area ratio to what is expected in healthy controls.

Treatment of BD with steroids and colchicine is associated with an increase in cytoplasmic area, but not to an amount that can bring the cell to a size approaching the healthy control value. In addition, treatment seems to disturb the originally preserved ratio in untreated BD cases.

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