

Hematological and cytomorphometric assessment in recurrent aphthous minor - A comparative study

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Key words

Cell area, cell diameter, complete blood cell count, nuclear area, nuclear diameter, recurrent aphthous stomatitis minor

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Abstract

Background: Recurrent aphthous stomatitis (RAS) is the common oral disease, characterized by the development of painful, recurring solitary or multiple ulcerations of the oral mucosa. There is no single satisfactory tool for diagnosis of RAS. In addition, there is a paucity of reports on cytomorphometric features in RAS.

Aims and Objectives: The current study had aimed at assessing cytomorphometric features in RAS minor patients and in healthy volunteers, keeping the baseline parameters of complete blood cell count. The objectives were to assess and compare cytopathological and cytomorphometric features in RAS minor and normal individuals.

Materials and Methods: Prospective study sample had comprised 15 cases of RAS minor and 15 of healthy volunteers. The complete blood cell count was performed; oral smears were prepared and were stained with Papanicolaou stain from all study subjects. Cytomorphometric parameters were compared between RAS minor and healthy volunteers. Student's *t*-test and SPSS 15.0 software were used for statistical analysis.

Results: On comparison, significant changes in nuclear area and in cell diameter were evident between RAS minor patients and healthy volunteers.

Conclusion: Cytomorphometric analysis may be considered as a valuable adjunct in the diagnosis of RAS minor.

Introduction

Recurrent aphthous stomatitis (RAS) is the most common oral disease, characterized by the development of painful, recurring solitary or multiple ulcerations of the oral mucosa.^[1,2] The reported prevalence in the general population varies from 5% to 66% with a mean of 20%.^[1] RAS has been classified chiefly into four varieties based on clinical manifestations as minor, major, herpetiform, and recurrent ulcers associated with Behcet's syndrome.

Minor aphthous ulcerations are the most common, accounting for 80% of all cases. Non-keratinized mucosal surfaces such as labial mucosa, buccal mucosa, and floor of the mouth are most commonly affected; ulcers do vary from 8 to 10 mm in size and they heal within 10–14 days without scarring.^[3] Recurrent aphthous major accounts for 10–15% of patients and it also affects non-keratinized mucosa; the ulcers persist for up to 6 weeks and then heal with scarring.^[3]

Herpetiform variant of RAS is characterized by recurrent multiple ulcers, which may be up to 100 in number; these ulcers may last for about 10–14 days.^[3]

Behcet's syndrome is a chronic disorder, characterized by oral and genital ulcers, arthritis, ocular, gastrointestinal, and neurological manifestations.^[2]

The etiopathogenesis of RAS so far remains incompletely understood; the triggering factors include Genetic predisposition, vitamin deficiencies, food allergies, viral and bacterial infections, systemic diseases (e.g., celiac disease, Crohn's disease, ulcerative colitis, and AIDS), increased oxidative stress, mechanical injuries, hormonal disturbances, and anxiety.^[4]

Even though RAS is proposed to be a multifactorial lesion, exact etiology is still unknown.^[3]

The microscopy of aphthous ulcer is nonspecific. Cytological examination would reveal neutrophils, lymphocytes along with few erythrocytes. Some cases exhibit cells with elongated nuclei containing linear bar of chromatin radiating toward nuclear membrane referred to as Anitschkow cells.^[2]

A single specific laboratory procedure is not yet available for a definitive diagnosis of RAS. Cytomorphometry is the quantitative measurement of cellular and nuclear features,

putting in data into a computer through a graphical interface with the purpose of standardizing image analysis.^[5]

The cytomorphometric analysis has been carried out on oral epithelial cells under normal and disease conditions.^[6-8]

RAS has shown a strong association with hematinic deficiency such as that of Vitamin B12, folic acid, and iron.^[9] These hematinic deficiencies may be reflected in complete blood cell count.

Search of Scientific English literature (Pubmed and Google search with keywords cytomorphometric analysis, RAS) had revealed no data on cytomorphometric analysis on sporadic cases of RAS. Only one report in Behcet's disease has shown cytomorphometric changes in oral exfoliated smears by Diyarbakir *et al.*^[10] Therefore, the present study had aimed at assessing the hematological parameters (Hb estimation, total red blood cell count, platelets, total white blood cell (WBC) count, differential leukocyte count, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and differential WBC count) and cytomorphometric characteristics in patients with recurrent aphthous minor. By hematological and cytological assessment, the present study had further aimed at throwing light on etiopathogenesis of RAS minor; cytomorphometry being used as a diagnostic adjunct.

Materials and Methods

A total of 30 prospective cases ($n = 30$; 15 cases of normal oral mucosa and 15 cases of RAS minor) chosen from the Outpatient Department of DAPM RV Dental College and Hospital, Bengaluru, had constituted the study material.

Oral smear samples were prepared from the healthy volunteers; in addition, smears were obtained from aphthous ulcer margin, healed, and contralateral areas of RAS minor patients. The written informed consent was taken from all the study subjects.

The age of the RAS minor patients had ranged from 17 to 35 years (5 males, 10 females); whereas, the age of normal healthy volunteers was again between 17 and 35 years (6 males, 9 females). All the smears were taken from lower labial mucosa for both the study groups.

Processing and interpretation of oral smears from study subjects were carried out in the Department of Oral and Maxillofacial Pathology, DAPM RV Dental College and Hospital, Bengaluru.

Methodology

Any three major and any two minor criteria (a total of five criteria) were considered for clinical diagnosis of RAS minor (Natah *et al.* and coinvestigators).^[12]

Control group: Blood sample was obtained to carry out complete blood cell count, random blood sugar test, and erythrocyte sedimentation rate (ESR) so as to exclude patients with hematologic disorders and to include only those individuals with normal blood cell count. Cytological smears were prepared

from normal lower labial mucosa using Cytobrush and the smears were fixed immediately with ethyl alcohol (fixative). The cytological smears were stained with modified Papanicolaou (PAP) staining technique. The microscopic examination of the smears was done under the bright field research microscope and photomicrographs were taken using Prog Res software. Slides were first scanned under $\times 4$ objective, followed by observation under $\times 10$. Under $\times 40$ objective, the slides were moved in a zigzag manner; one cell per field was selected without any overlap and the images were captured. A total of 10 fields were selected and the images were captured for 10 individual cells. The captured cells were subjected to cytomorphometric analysis by Image J software. Nuclear diameter, nuclear area, cellular diameter, and cellular area were measured [Figure 1] using Image J software. Nuclear-cytoplasmic ratio was calculated using the formula. The data were tabulated in a master chart for further analysis and interpretation.

RAS group: Complete blood cell count, random blood sugar test, and ESR were carried out to screen hematological disorders. Individuals with normal blood cell count were included for the present study. From the same patients, cytological smears were prepared from aphthous ulcer (lower labial mucosa) margin [Figure 2] and contralateral areas using Cytobrush. The smears were fixed immediately with ethyl alcohol (fixative). The cytological smears were stained with modified PAP staining method. The microscopic examination and morphometric analysis of the smears were done in a similar pattern as described for normal oral mucosal smears. Patients were recalled after 10–15 days to check for healing of the ulcer. When the ulcer had healed, a smear was prepared from the healed area; it was fixed and was stained with PAP stain. A total of 45 smears were taken from RAS minor patients, out of which 15 were taken from ulcer margin, another 15 from contralateral side without ulcer, and the remaining 15 were from the healed area. The same protocol as mentioned before was repeated for these smears as well [Figures 2-5].

The data were tabulated in a master chart for further analysis and interpretation.



Figure 1: Clinical photograph showing recurrent aphthous minor in the lower labial mucosa

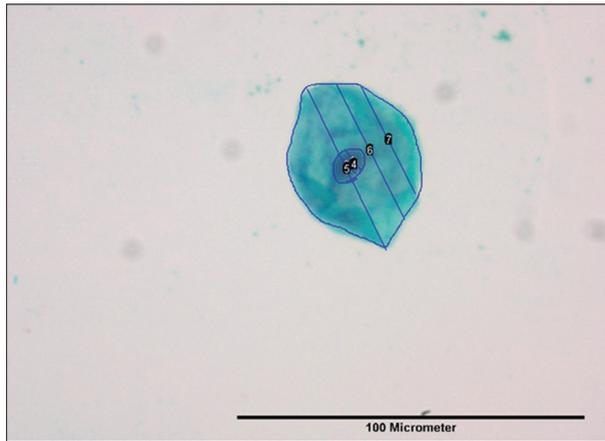


Figure 2: Photomicrograph of the smear from control group with cytomorphometric analysis (Papanicolaou stain $\times 400$; Image J 1.47v software java 1.6.0_20 [32-bit]) 1: Nuclear area; 2 and 3: Nuclear diameter; 4: Cellular area; 5, 6, and 7: Cellular diameter

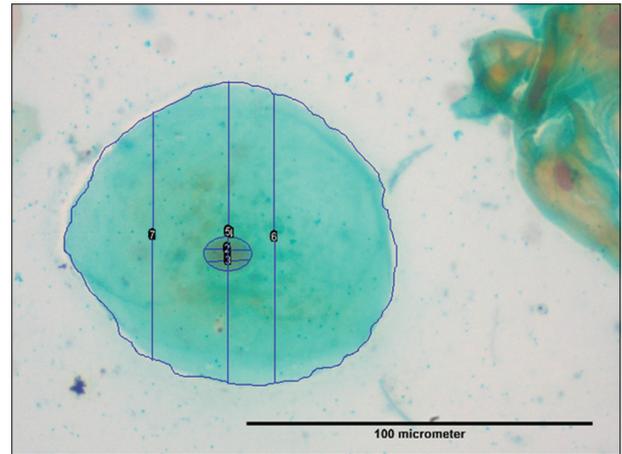


Figure 4: Photomicrograph of the smear from recurrent aphthous stomatitis minor (healed area) with cytomorphometric analysis (Papanicolaou stain $\times 400$; Image J 1.47v software java 1.6.0_20 [32-bit]) 1: Nuclear area; 2 and 3: Nuclear diameter; 4: Cellular area; 5, 6, and 7: Cellular diameter

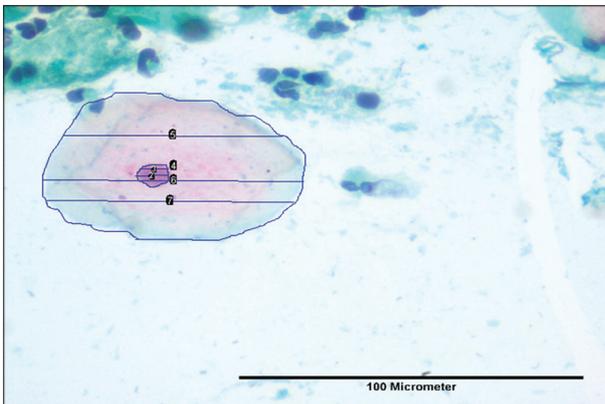


Figure 3: Photomicrograph of the smear from recurrent aphthous stomatitis minor (ulcer area) with cytomorphometric analysis (Papanicolaou stain $\times 400$; Image J 1.47v software java 1.6.0_20 [32-bit]) 1: Nuclear area; 2 and 3: Nuclear diameter; 4: Cellular area; 5, 6, and 7: Cellular diameter

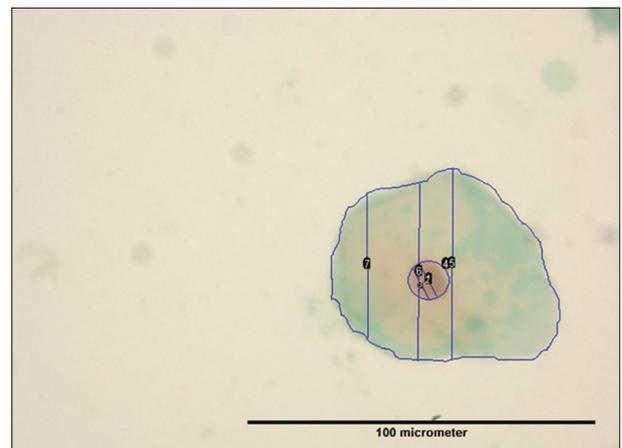


Figure 5: Photomicrograph of the smear from recurrent aphthous stomatitis minor (contralateral area) with cytomorphometric analysis (Papanicolaou stain $\times 400$; Image J 1.47v software java 1.6.0_20 [32-bit]) 1: Nuclear area; 2 and 3: Nuclear diameter; 4: Cellular area; 5, 6, and 7: Cellular diameter

Wherever required, strict aseptic precautions were maintained; confidentiality of the patient details was preserved.

Statistical methods

Descriptive and inferential statistical analyses had been carried out for the present study. Results on continuous measurements were presented on mean \pm standard deviation (min-max) and results on categorical measurements were presented in number (%). Significance was assessed at 5% level of significance. The following assumptions on data were made, Assumptions: (1) Dependent variables should be normally distributed and (2) samples drawn from the population should be random, cases of the samples should be independent.

Student's *t*-test (two-tailed, independent) had been used to find the significance of study parameters on continuous scale

between two groups (intergroup analysis) on metric parameters. LevenIs test for homogeneity of variance had been performed to assess the homogeneity of variance.

Statistical software

The Statistical software, namely, SAS 9.2, SPSS 15.0, Stata 10.1, MedCalc 9.0.1, Systat 12.0, and R environment ver.2.11.1 was used for the analysis of the data and Microsoft Word and Excel had been used to generate graphs and tables.

Results

A total of 30 cases ($n = 30$) had constituted the study group.

Cytology of normal oral mucosa

The PAP stained cytological smears had shown normal squamous epithelial cells. There was no evidence suggestive of any pathology.

Cytopathology in RAS

The PAP stained cytological smears had shown many squamous epithelial cells. Moderate to abundant mixed inflammatory infiltrate (neutrophils and lymphocytes) was seen. There was no evidence of altered nuclear chromatin in these cells (suggestive of Anitschkow cells). Cytological features in RAS minor patients were suggestive of non-specific inflammation.

Cytomorphometric analysis

Comparison of cytomorphometric parameters between ulcer margin of RAS minor and control group

The nuclear area was higher in RAS minor group when compared to that of control group. This difference was statistically significant [Table 1]. The cell area, cell diameter, and nuclear-cytoplasmic ratio were decreased in RAS minor group when compared to that of control group. These differences were also statistically significant [Table 1].

Comparison of cytomorphometric parameters between ulcer margin of RAS minor and contralateral area of RAS minor group

The nuclear area was higher in RAS minor group when compared to that of contralateral area of RAS minor. This difference was statistically significant [Table 2]. The cell area, cell diameter, and nuclear-cytoplasmic ratio were decreased in ulcer margin of RAS minor group when compared to that of contralateral area of RAS minor. These differences were again statistically significant [Table 2].

Comparison of cytomorphometric parameters between healed area of RAS minor and ulcer area of RAS minor group

The nuclear area was higher in ulcer margin of RAS minor group when compared to that of healed area of RAS minor. This difference was statistically significant [Table 3]. The cell area, cell diameter, and nuclear-cytoplasmic ratio were decreased in ulcer area of RAS minor group when compared to that of healed area of RAS minor. These differences were also statistically significant [Table 3].

Comparison of cytomorphometric parameters between healed area of RAS minor and control group

All the parameters were normal except increased nuclear-cytoplasmic ratio in healed area of RAS minor when compared to that of control group. This difference was statistically significant [Table 4].

Comparison of cytomorphometric parameters between contralateral area of RAS minor and healed area of RAS minor

The cell diameter was increased in healed area in RAS minor when compared to contralateral area of RAS minor. This difference was statistically significant [Table 5].

Comparison of cytomorphometric parameters between contralateral area of RAS minor and control group

Only cell diameter had reduced in contralateral area of RAS minor when compared to control group, which was statistically significant as shown in Table 6.

Discussion

RAS represents one of the common pathologies affecting oral mucous membrane. The etiopathogenesis of these recurrent aphthous ulcers has been debated from quite a long

Table 1: Comparison of study variables in two groups studied

Variables	Normal	Ulcer	P
Nuclear area	74.84±15.88	94.24±14.29	0.002**
Nuclear diameter	10.61±1.27	10.15±0.96	0.277
Cell area	3291.45±504.11	2804.44±295.24	0.003**
Cell diameter	70.85±8.03	55.51±3.57	<0.001**
Nuclear-cytoplasmic ratio	0.235±0.005	0.0354±0.007	<0.001**

SD: Standard deviation, **: Strongly significant (P value: P≤0.01)

Table 2: Comparison of study variables in two groups studied

Variables	Contralateral	Ulcer	P
Nuclear area	79.87±12.83	94.24±14.29	0.007**
Nuclear diameter	10.34±0.89	10.15±0.96	0.597
Cell area	3133.21±414.97	2804.44±295.24	0.019*
Cell diameter	62.90±5.58	55.51±3.57	<0.001**
Nuclear-cytoplasmic ratio	0.0263±0.005	0.0354±0.007	0.001**

SD: Standard deviation, *: Moderately significant (P value: 0.01<P≤0.05),

** : Strongly significant (P value: P≤0.01)

Table 3: Comparison of study variables in two groups studied

Variables	Healed	Ulcer	P
Nuclear area	81.96±12.99	94.24±14.29	0.020*
Nuclear diameter	10.70±0.85	10.15±0.96	0.115
Cell area	3179.5±392.39	2804.44±295.24	0.006**
Cell diameter	70.02±3.85	55.51±3.57	<0.001**
Nuclear-cytoplasmic ratio	0.0267±0.004	0.0354±0.007	0.001**

SD: Standard deviation, *: Moderately significant (P value: 0.01<P≤0.05),

** : Strongly significant (P value: P≤0.01)

Table 4: Comparison of study variables in two groups studied

Variables	Normal	Healed	P
Nuclear area	74.84±15.88	81.96±12.99	0.190
Nuclear diameter	10.61±1.27	10.70±0.85	0.832
Cell area	3291.45±504.11	3179.50±392.39	0.503
Cell diameter	70.85±8.03	70.02±3.85	0.723
Nuclear-cytoplasmic ratio	0.235±0.005	0.0267±0.004	0.083+

SD: Standard deviation, +: Suggestive significance (P value: 0.05<P<0.10)

Table 5: Comparison of study variables in two groups studied

Variables	Contralateral	Healed	P
Nuclear area	79.87±12.83	81.96±12.99	0.660
Nuclear diameter	10.34±0.89	10.70±0.85	0.269
Cell area	3133.21±414.97	3179.50±392.39	0.756
Cell diameter	62.90±5.58	70.02±3.85	<0.001**
Nuclear-cytoplasmic ratio	0.0263±0.005	0.0267±0.004	0.829

SD: Standard deviation, **: Strongly significant (P value: $P \leq 0.01$)

Table 6: Comparison of study variables in two groups studied

Variables	Normal	Contralateral	P
Nuclear area	74.84±15.88	79.87±12.83	0.348
Nuclear diameter	10.61±1.27	10.34±0.89	0.498
Cell area	3291.45±504.11	3133.21±414.97	0.356
Cell diameter	70.85±8.03	62.90±5.58	0.004**
Nuclear-cytoplasmic ratio	0.0235±0.005	0.0263±0.005	0.122

SD: Standard deviation, **: Strongly significant (P value: $P \leq 0.01$)

time. Various proposed triggering factors include nutritional deficiencies, microbial infections, genetic factors, altered redox balance, hormonal disturbances, anxiety, and others. Although many investigative devices have been explored, there is lack of a specific diagnostic tool for RAS. Clinical examination along with microscopic correlation remains the standard protocol. However, microscopy alone remains inconclusive.

Scientific English literature has shown association of hematinic deficiencies in some patients of RAS and correction of these deficiencies had resulted in prompt relief.^[1-13]

The present study had aimed at hematological and cytological assessment in RAS minor and in normal subjects. Oral mucosal smears were studied from 15 RAS minor patients and from 15 healthy volunteers. On comparison, significant changes in nuclear area and in cell diameter were evident between RAS minor patients and healthy volunteers.

The present study is first of its kind in assessing cytomorphometric parameters in recurrent aphthous minor patients without any systemic manifestations.

In a study by Diyarbakir *et al.*, the nuclear volume and cytoplasmic volume of the oral mucosal cells (buccal mucosa and floor of the mouth) were higher in patients with Behcet's disease.^[10] In the present study, the nuclear area of the cells from ulcer margin of RAS had shown higher value when compared to that in healthy oral mucosal smears.

Another cytomorphometric study on buccal mucosal cells of Behcet's disease was reported by Aktunc *et al.*^[13] The authors had observed that nuclear area of the cells was significantly smaller when compared to that in healthy volunteers; the nuclear-cytoplasmic ratio did not differ between the groups. In contrast, the present study had revealed increase in nuclear area in ulcer margin of RAS minor and increase in nuclear-cytoplasmic ratio in healed area of RAS minor patients when compared to healthy oral mucosal smears. The increase in nuclear area from cells of

ulcer margin could be reflecting the cellular response secondary to redox imbalance and or due to upregulation of heat shock proteins as a consequence of local inflammatory reaction. The increase in nuclear-cytoplasmic ratio observed in healed areas of RAS minor could be due to incomplete maturation of the epithelial cells during the healing process.

There are no reported studies on the cytomorphometric changes in healed and contralateral areas of RAS minor patients. Therefore, the present study is the first of its kind to assess the cytomorphometric changes at various phases in sporadic cases of RAS minor and to compare those with normal oral mucosal smears.

Conclusion

The current study has shown significant cytomorphometric changes in recurrent aphthous minor on comparison with healthy oral mucosal smears. Thus, cytomorphometry could be used as an adjunct in the diagnosis of RAS minor; however, the study is being continued on a larger sample, the report of which will appear in the next paper.

Clinical Significance

The cytomorphometry may be used as a diagnostic adjunct for RAS minor.

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