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Evaluation And Comparison Of Micronuclei In Exfoliative Cytology Of Buccal Mucosa In Normal Individuals And Sickle Cell Anemia Patients Of Durg City Of Chhattishgarh State

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Abstract

Context: The mouth and face are highly accessible parts of the body, sensitive to and able to reflect changes occurring internally. In order to contribute the understanding of the role of the different biomarkers and their relationship with the extremely variable clinical manifestation of sickle cells disease, this study investigated the micronuclei frequency in buccal cells in sickle cell traits, sickle cell anemia patients and controls.

Aims: To assess the frequency of micronuclei in buccal cells of patients with sickle cell trait, sickle cell disease and healthy individuals.

Settings and Design: The sample for present study comprised of 30 patients of Sickle cell trait , 30 patients of sickle cell disease and 30 healthy individuals as control .

Methods and Material: .Exfoliative cytology slides were prepared from buccal mucosa. Cells were examined and micronuclei frequency was calculated in percentage for each subject.

Statistical analysis used: Results were evaluated for statistical significance using student t test unpaired and ANOVA test

Results: For group I of sickle cell trait mean micronucleated cell % observed was 0.12 with standard deviation (SD) of 0.13. For group II (sickle cell disease) Mean micronucleated cell % observed was 0.75 with a SD of 0.22 for group III (control) mean micronucleated cell % was 0.08 with SD of 0.11.

Conclusions: The study suggests that individuals with sickle cell anemia, regardless of clinically visible oral lesions, show cytological changes in oral mucosal epithelium.

Key-words: Sickle cell anemia ,exfoliative cytology , micronuclei.

Key message: The study suggests that individuals with sickle cell anemia, regardless of clinically visible oral lesions, show cytological changes in oral mucosal epithelium. Results suggest that formation of micronucleus is highly associate with sickle cell disease.

Introduction:

Sickle cell disease (SCD) is a class of hemoglobinopathy, which results from a single mutation in the β-globin chain inducing the substitution of valine for glutamic acid at the sixth amino acid position . This mutation leads to the production of abnormal hemoglobin (hemoglobin S [HbS]). In addition to homozygous sickle cell disease (HbSS)[1].

The sickle cell gene occurs throughout Indian society although it is most common among the tribal people, who are predominantly agricultural and often live in remote areas, presenting particular problems in provision of health care and education. Sickle cell hemoglobinopathy is a common health problem in Chhattisgarh, out of 26 million population nearly 27% of population suffered with heterozygous hemoglobin trait and 2.5% with fatal homozygous hemoglobindisease , nevertheless relatively few studies describe the prevalence and demographic patterns of people with sickle cell disease in this region.[2]

Changes in teeth and oral mucosa are due to local diseases and systemic diseases wide variety of oral manifestation occurs in various systemic diseases. Diseases of blood frequently affect soft and hard tissues of mouth with different characteristics. Oral manifestation of blood disease affect color of the mucosa hypertrophy of gingiva , mucosal destruction in the form of ulceration ,bleeding and hemorrhage, color of the tooth with red discoloration , lymph node and affect the bone with decreased density and enlarged marrow space.[3]

It is well established that micronuclei are formed from the entire chromosome or from a fragment of it . Such micronuclei are induced by genotoxic stress such as clastogen or aneugen. Micronuclei induction by clastogen involves the induction of either Chromosome fragments that lag behind the separating chromosomes .[4]

Exfoliative cytology is the microscopic examination of shed or desquamated cells from the epithelial surface usually the mucous membrane. It also includes the study of those cells that have been collected by scraping the tissue surface or collected from body fluids such as sputum, saliva, etc.

The buccal cell (BC) micronucleus (MN) assay, first proposed by Stich et al is useful as a biomarker of genetic damage caused by life-style habits (e.g. smoking consumption and/or alcohol micronutrient deficiency) and of exposure to environmental pollutants as well as in patients with inherited genetic defects in DNA repair .[5]

Studies on the frequency of micronuclei existence in buccal cells from patients with sickle cell anemia have not been reported so far. In order to contribute the understanding of the role of the different biomarkers and their relationship with the extremely variable clinical manifestation of sickle cells disease, this study investigated the MNs frequency in buccal cells in sickle cell traits, sickle cell anemia patients and controls.[1]

Subjects and Methods:

Patients for the study were selected from regular outpatient department of oral and maxillofacial pathology MCDRC, Anjora, Durg, OPD and hematology department of district hospital Durg Chhattisgarh.The study was approved from the ethical committee of the institution. The participants were explained about the study and then included with an informed consent.

Study Group:

The sample for present study comprised of 30 patients of Sickle cell trait , 30 patients of sickle cell disease and 30 healthy individuals as control . Patients who are healthy, i.e. not suffering from any other systemic diseases and were willing to take part in the study were included in the study. Patients suffering from any systemic illness including diabetes mellitus, cardiovascular, liver and renal disease and those with smoking habit, tobacco chewing and alcohol intake habit.were excluded from the study. Also individuals with premalignant lesion or condition or carcinoma were excluded.

Source and method of collection of data:

The subjects in this study were categorized as Group I 30 Patients of sickle cell anemia trait.

Group II 30 Patients of sickle cell disease and Group III 30 normal individuals as control group.

The subjects included in the study were randomly selected from the sickle cell unit at Durg district hospital. Written consent was obtained from the patients. After explaining about the study to the subjects , an informed consent was obtained . a detailed history with thorough clinical examination was done and findings

were recorded in a specially prepared case history proforma. The proven cases of sickle cell disease and sickle cell trait were included in the study. All the subjects were diagnosed cases at district hospital Durg and by gel electrophoresis under sickle cell screening project, Biochemistry department JNM Medical college Raipur. Control patients were screened by solubility test in departmental hematology laboratory.
Exfoliative cytology procedure was performed and slides prepared were stained using PAP stain .The most commonly used method, i.e. the zig-zag method, was followed for screening of slides. Total 1000 cells were counted per slide and micronuclei frequency was calculated in percentage for each subject. [6,7]
The criteria developed by Tolbert et al. for choosing the cells are the most widely used. They consist of the following parameters for cell inclusion in the cells to be scored: [8]
Intact cytoplasm and relatively flat cell position on the slide; little or no overlap with adjacent cells;
little or no debris; nucleus normal and intact; nuclear perimeter smooth and distinct.
The suggested criteria for identifying MN are: rounded smooth perimeter suggestive of a membrane; less than a third the diameter of the associated nucleus, but large enough to discern shape and color; Feulgen positive, i.e. pink in bright field illumination; staining intensity similar to that of the nucleus; texture similar to that of nucleus; same focal plane as nucleus; and (g) absence of overlap with, or bridge to, the nucleus. (Figure: 1)

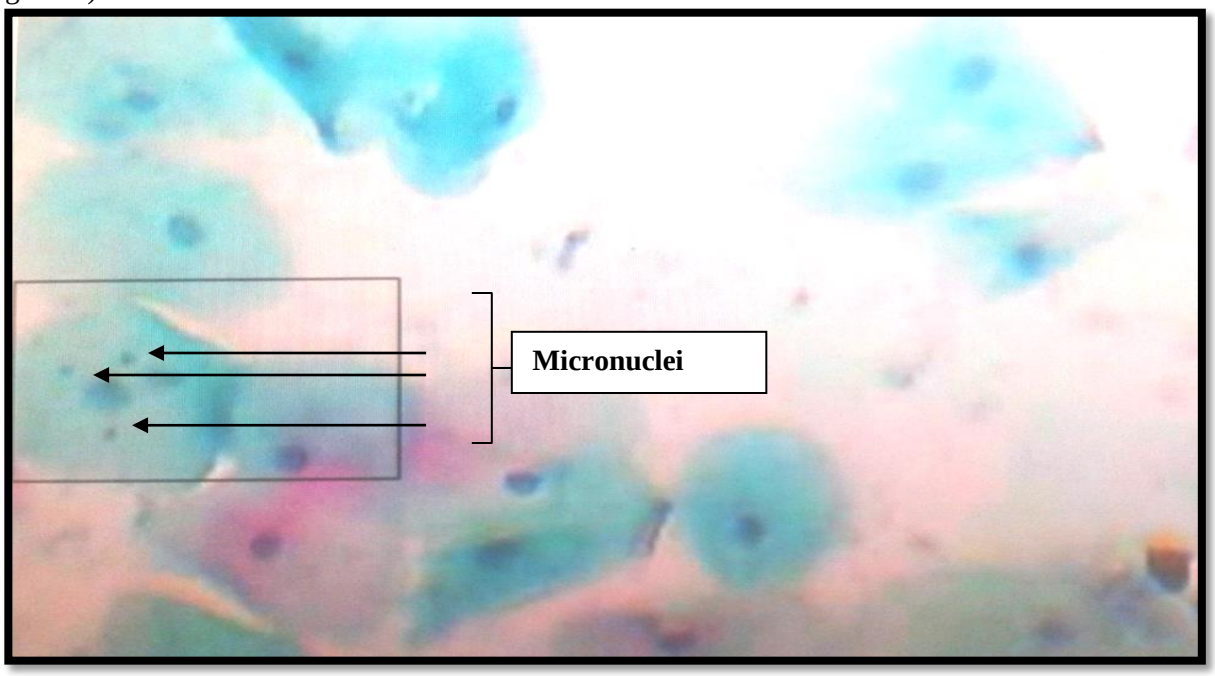


Figure: 1
Armamentarium for examination and exfoliative cytology:
Mouth mirror, Mouth mask, sterile gloves, written informed consent, wooden tongue spatula, Glass slide, 95% isopropyl alcohol as spray fixative.
Equipment and materials used for pap staining:
Rapid PAP stain kit , Rapid PAP stain kit , Coplin jars , Cover slip (22 x 25 mm, 0.16 mm thick) , DPX
Equipment for microscopy and image capture:
Labomed (LX 400) trinocular microscope. Digital camera (Labomed) with Pixel Pro software.
Results were evaluated for statistical significance using student t test unpaired and ANOVA test.

Results:

Table 1

Age wise distribution of subjects studied

Group (Age)	MEAN	SD	Significance
Group I (Sickle Cell Trait)	13.37	3.76	p>0.05
Group II (Sickle Cell Disease)	10.03	4.18	
Group III (Control)	9.96	2.14	

Graph 1

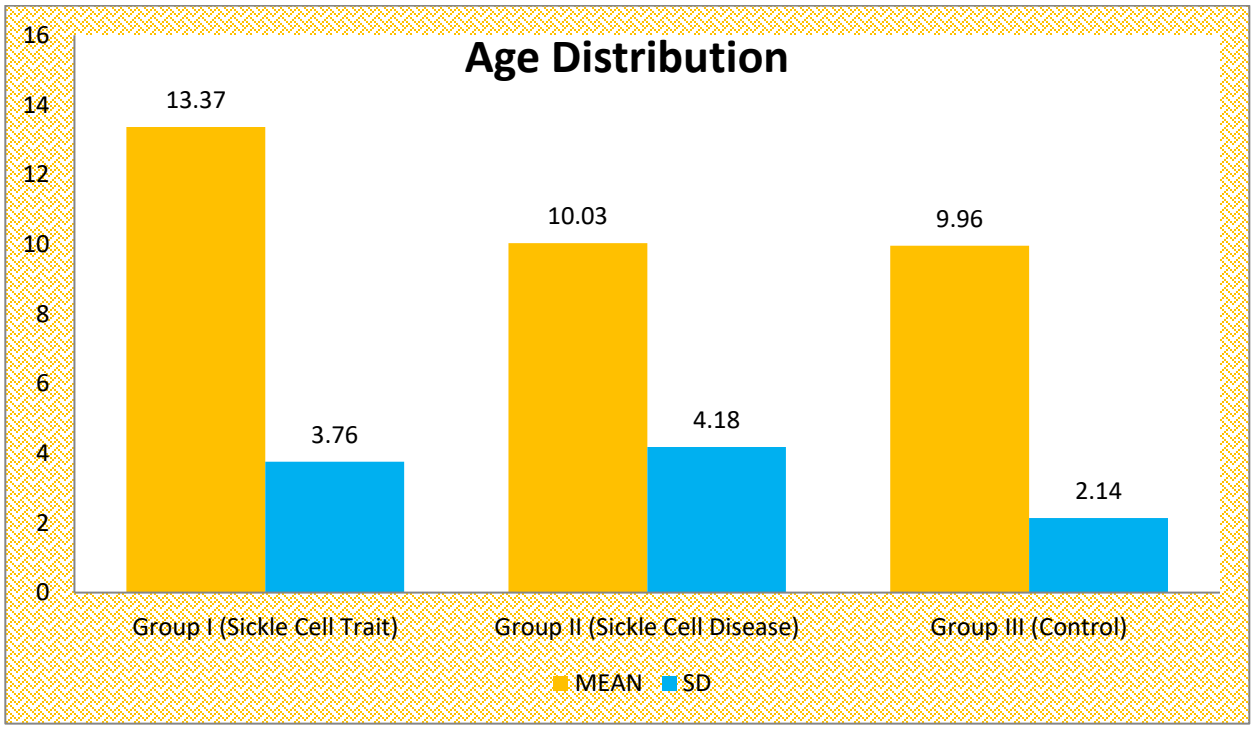
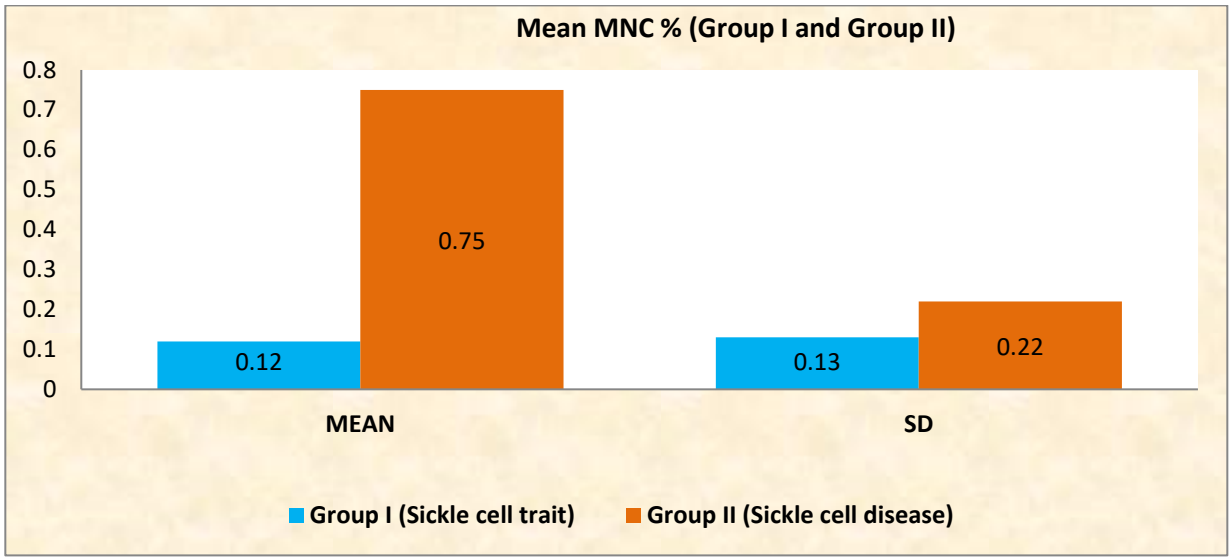


Table: 2

Micronucleus frequency comparison between **Group I** and Group **II**

Group	MEAN	SD	SIGNIFICANCE
Group I (Sickle cell trait)	0.12	0.13	P=0.0001 HS
Group II (Sickle cell disease)	0.75	0.22	

Graph: 2

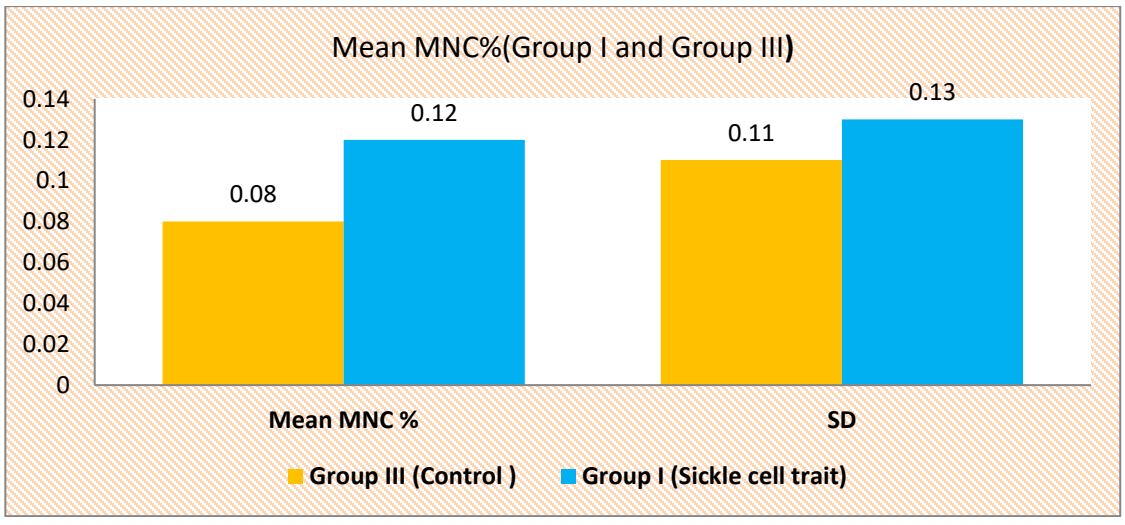


The above table and graph shows comparison of mean MNC % between Group I and Group II There is significant difference between the mean MNC %.(P<0.0001) The Unpaired student t test shows p value of 0.0001 which is statistically highly significant.
Observed difference in mean is statistically significant at P<0.0001

Table: 3
Micronucleus frequency comparison between Group I and Group III

GROUP	Mean MNC %	SD	SIGNIFICANCE
Group III (Control)	0.08	0.11	p=0.205 NS
Group I (Sickle cell trait)	0.12	0.13	

Graph: 3

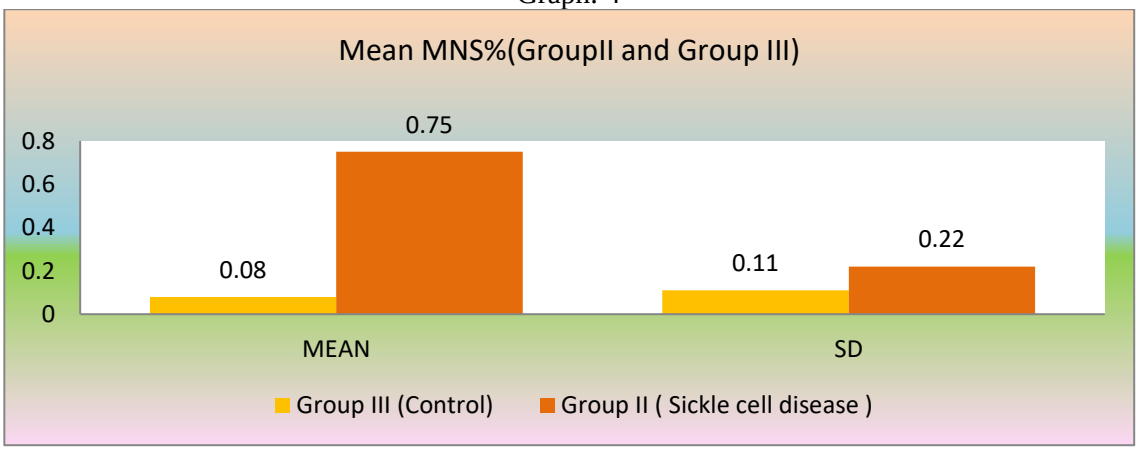


The above table and graph shows comparison of mean MNC% between Group III and Group I patients. There is no significant difference between the mean MNC %. The student t test shows p value of 0.58 at a level of significance 0.05 which is statistically not significant.
Observed Difference in means is not statistically significant (P>0.05)

Table 4: Micronucleus Frequency comparison between Group II and Group III

Group	MEAN	SD	SIGNIFICANCE
Group III (Control)	0.08	0.11	P=0.0001 HS
Group II (Sickle cell disease)	0.75	0.22	

Graph: 4



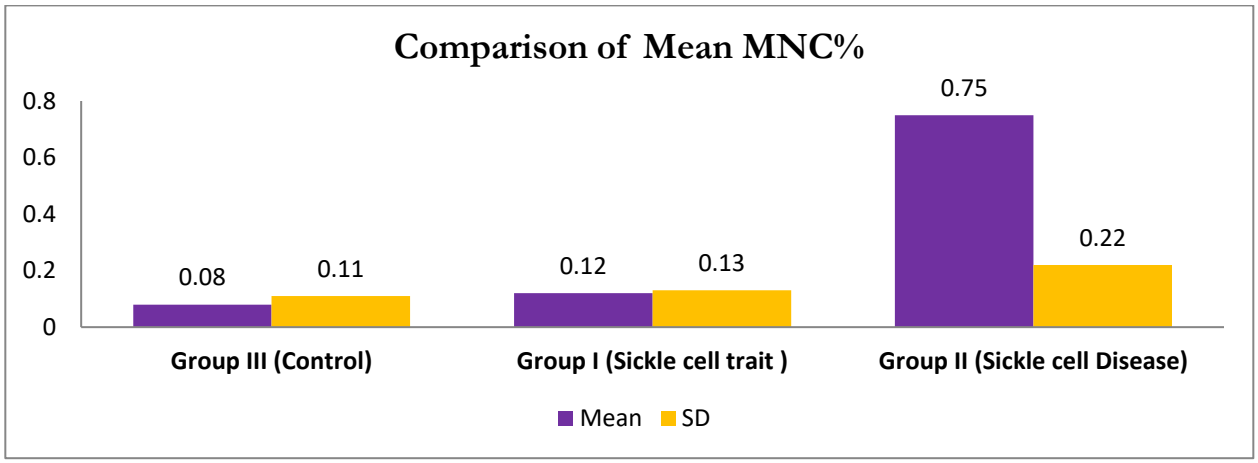
The above table and graph shows comparison of mean MNC % between Group III and Group II. There is significant difference between the mean MNC %. The student t test shows p value of 0.0001 at a level of significance 0.05 which is statistically highly significant. Difference in mean observed is statistically significant at P<0.0001

Table: 5

	Group III (Control)	Group I (Sickle cell trait)	Group II (Sickle cell Disease)	Significance by Anova test
Mean	0.08	0.12	0.75	P<0.0001 HS
SD	0.11	0.13	0.22	

Intergroup comparison of micronuclei frequency

Graph: 5



All three groups were compared using one way ANOVA test and the p value was< 0.0001 which is statistically highly significant.
Difference in mean observed is statistically significant at P<0.0001.

Discussion:

The objective of this study was to assay the genomes' stability in patients with sickle cell anaemia by a micronucleus assay in buccal mucosa cells.[10]Studies on frequency of micronuclei existence in buccal cells from patients with sickle cell anemia have not been reported much so far. In order to contribute the understanding of the role of the different biomarkers and their relationship with the extremely variable clinical manifestation of sickle cell disease, we investigated the micronuclei frequency in buccal cells in sickle cell traits, sickle cell anemia patients and controls. We found an increased frequency of micronuclei in sickle patients when compared with control subjects. The purpose of this study was to evaluate the cells obtained from buccal scrapings of subjects with sickle cell disease, sickle cell trait and control.

Buccal cells are the first barrier for the inhalation or ingestion route and are capable of metabolizing proximate carcinogens to reactive products. Approximately 90% of human cancers originate from epithelial cells. Therefore, it could be argued that oral epithelial cells represent a preferred target site for early genotoxic events induced by carcinogenic agents entering the body via inhalation and ingestion.[8]

The mean micronucleated cell % was calculated in our study after observing the buccal smear of 30 cases in each group. A total of 1000 cells were observed per slide. For group I of sickle cell trait mean micronucleated cell % observed was 0.12 with standard deviation of 0.13. For group II (sickle cell disease) Mean micronucleated cell % observed was 0.75 with a standard deviation of 0.22 for group III (control) mean micronucleated cell % was 0.08 with standard deviation of 0.11.

Results of this study when compared with **Shubber *et al*** who evaluated micronucleus frequencies in buccal cells from patients with sickle cell anemia showed similar results. Cells with micronuclei were significantly higher i.e 4.2MN/1000 cells when compared to control group 0.65N in Shubber*et al* study except for sickle cell trait group . Sickle cell trait group in our study showed no significant difference in mean micronucleated cell % (0.12) when compared with control (0.08) was statistically insignificant. While in the former study it showed double the value of baseline.

Our study on comparision with **Granvil L. Hays (1977)** who studied nuclear chatracterstics of buccal mucosa cells in sickle cell anemia and concluded that no buccal nuclear forms are peculiar to sickle cell anemia alone. They also concluded that an increase in the number of cells exhibiting aberrations, other than just an occasional cell indicates a potentially anemic patient. Since our study did not involve different types of anemia so this fact could not be studied.

This study on comparison with **Diversi Hl *et al*** who performed correlation of cytologic nuclear changes to anemias our study showed similar results i.e. increase in number of cells exhibiting nuclear aberration.

Our study is in agreement with **Parizo *et al* (2013)** who in a study performed cytomorphometric and cytomorphologic analysis of oral mucosa in childern with sickle cell anemia and concluded that nuclear

aberrartions of epithelial cells in oral mucosa have been demonstrated in patients with sickle cell anemia , pernicious and iron deficiency anemia. However these findings may not be pathognomic for a specific type of anemia or for any anemia. Morphologic changes were observed in this study with a highly significant increase in nuclear aberration in sickle cell anemia group.[53] However our study did not consider different types of anemia and it considered miconuclei frequency as the marker for nuclear aberrations.

Causes of the presence of micronuclei in buccal cells from sickle cell anemia patients are unknown. It was reported that sickle cell anemia patients are subjected to increased oxidative stress particularly during vaso-occlusive crises and acute chest pain. Another possible cause of oxidative stress in sickle cell disease is the high concentration of iron in the patient's plasma. The increase in oxidative stress could be a relevant risk factor for mutagenesis and carcinogenesis. Moreover, patients with sickle cell anemia often are suffering from folic acid and vitamin B_{12} depletion because of their chronic haemolytic anemia. Folic acid and B_{12} play a critical role in the prevention of chromosome breakage that leads to micronucleus formation. Both in vitro and in vivo studies with human cells clearly show that folate B_{12} deficiency causes expression of chromosomal fragile sites, chromosome breaks, excessive uracil in DNA, micronucleus formation and DNA hypermethylation. [9]

It is well established that reduction of blood supply, inadequate nutrition, and the loss of innervations are the most common causes of oral epithelium atrophy. Sickle cell anemia patients are more susceptible to develop oral epithelium atrophy due to the pathophysiology of the disease that includes anemia and vasculopathy, resulting in microvascular obstruction by sickle cells, preventing blood flow to tissue. Oral epithelium atrophy may affect the balance between protein synthesis and degradation, giving rise to abnormalities in cell structure and keratinization pattern of the oral epithelium. Other cause of oral epithelial atrophy is the deficiency of B_{12} vitamins that is necessary for the synthesis of red blood cells.

The study suggests that individuals with sickle cell anemia, regardless of clinically visible oral lesions, show cytological changes in oral mucosal epithelium. Several difficulties must still be overcome before the micronucleus assay on exfoliated cells can be considered as a validated method for the monitoring of sickle cell anaemiain humans. As exfoliated cells frequently undergo morphologic changes leading to cell death and nuclear disruption, it is of the utmost importance to distinguish between the degenerative nuclear phenomena and true micronuclei.

Micronucleus observed in buccal cells is not induced when the cells are at the epithelial surface but are formed in the basal layers. There was no significant difference between individuals, carrying single sickle cell gene and control groups in the presence of micronucleus in their buccalcells. These results may suggest that formation of micronucleus is highly associate with sickle cell disease severity i.e. presence of both SC genes. Such presence would lead to genome instability which resulted in micronucleus formation.

Children may be more susceptible to the effects of the environmental exposure and medical treatments than adults; however, limited information is available about the differences in genotoxic effects in children by age, sex and health status. Micronucleus assay is a well established method of monitoring genotoxicity, and this approach is thoroughly validated for adult lymphocytes by the Human Micronucleus Biomonitoring project similar international undertaking is in progress for exfoliated buccal cells.

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