CYTOLOGICAL GRADING OF ORAL SUBMUCOUS FIBROSIS

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ABSTRACT

Oral submucous fibrosis (OSMF) is a condition that was first described in the 1950s. It is caused as a result of addiction to harmful areca nut products with or without tobacco. The rationale of using exfoliative cytology in our study lies in the epithelial physiology where continuous exfoliation of epithelial cells is a part of physiological turnover. Deeper cells, which are strongly adhered in normal conditions, become loose in the case of malignancy and exfoliate along with superficial cells.

Our aim in this study was to compare the cellular changes such as formation of micronuclei within the cell and cytomorphometric analysis of the buccal mucosal cells of OSMF patients with that of normal controls.

We identified thirty three such cases of OSMF on the basis of oral inspection and examination. We used exfoliative cytology and liquid based cytogy to obtain buccal cells. The smear thus prepared was stained with feulgan fast green, acridine orange and papanicolou. Micronuclei were identified and cytomorphometric analysis was done using Adelta software.

There was a change in the hue of Papanicolou from pink to purplish indicating the degree of keratinization from normal cells to cells affected by OSMF. Acridine orange gave a green emission at wavelength 480-490 to normal cells, while it gave a bright red fluorescence in cells undergoing apoptosis. Mean cellular diameter decreased from normal-cells affected oral lesions. Mean nuclear cytoplasmic ratio increased from normal-cells to those affected by oral lesions. Frequency of micronuclei increased from normal to the cells affected by oral lesions.

Buccal cell mutations in premalignant and malignant lesions can serve as a useful tool for the bio-monitoring of oral lesions. Exfoliative Cytology being minimally invasive and cost effective can help in mass screening programmes.

KEYWORDS: Oral submucous fibrosis, Exfoliative cytology, Cytomorphometry, Micronuclei

INTRODUCTION

A number of changes can be observed within the oral cavity due to advancing age, due to environmental and certain life style related factors. Lesions of the oral cavity may occur as a result of trauma, infections, systemic diseases and due to excessive consumption of alcohol or smokeless tobacco (1). Oral lesions such as submucous fibrosis, lichen planus and leukoplakia are linked to certain habits such as chewing tobacco smoking and drinking (2). Many of these lesions have a tendency to transform into a malignant lesion and are thus termed as precursor lesions.

A premalignant/ precancerous lesion is a tissue with an altered morphology and has potential to change into a malignant lesion. A precancerous condition on the other hand is a state in which the risk of developing cancer is increased. In a World Health Organization (WHO) Workshop, held in the year 2005, a decision was made to use the term "potentially malignant disorders (PMD)" for all precursor lesions which means that all disorders described under this term may not have a tendency to convert into cancer (3). The following lesions such as Leukoplakia, Oral lichen planus, Erythroplakia, Oral submucous fibrosis (OSMF), Discoid lupus erythematosus, palatallesion of reverse cigar smoking have been identified as (PMD) Potentially Malignant Disorders by the World Health Organisation in association with Oral Cancer (4).

Exfoliative cytology was earlier used to detect cervical cancer cells Exfoliative cytology was first used for cervical cancer cells (5-9) but nowadays its usefulness has spread towards early detection of changes within the oral mucosa. Buccal epithelial cells are the first cells coming in contact with anything that is ingested or inhaled. These cells are therefore considered as the ideal site for examining genotoxic occurrences.

Buccal epithelial cells are exfoliated everyday (10). It is rare to find mitotic features in a cell during the last

stages of differentiation (11, 10). A Cytomorphometric examination of these cells is thus a quantitative method to examine the influence of smokeless tobacco consumption on the buccal mucosa. There are many developmental and degenerative diseases which are caused by genomic alterations. Some known genotoxins are as follows, certain medical procedures, micronutrient deficiencies, lifestyle factors and inherited defects in DNA metabolism (12,13), therefore it is of utmost importance that cheap, minimally invasive biomarkers are available. This will aid in early diagnosis thereby improving the prognosis as well as treatment of diseases caused by genetic damage. The micronuclei assay is an excellent method to serve as a biomarker which detects chromosomal loss or malfunction of mitotic spindles.

There is much controversy regarding the criteria that should be followed for identification and method of counting of micronuclei. Recent studies have attempted to establish a correlation between frequency of micronuclei found in oral exfoliated cells and the grading of oral carcinoma through histopathology.

AIMS AND OBJECTIVES

To study the oral mucosa of subjects by exfoliative cytology and to assess the cytological and nuclear changes therein for early detection of oral lesions thus helping in better treatment and prognosis.

MATERIAL AND METHOD

An epidemiological survey was conducted in randomly selected 12 villages between Lucknow and Kanpur. Villagers habituated to smokeless tobacco were screened for oral lesions. Two groups of 60 individuals each were formed, one group comprised of healthy controls while the other group of 60 participants were those with oral lesions. An informed consent was obtained from each individual. An oral screening was performed using toluidine blue for suspected lesions. Buccal smears of suspected lesions were obtained using exfoliative cytology.Before sampling, each subject was asked to rinse his mouth thoroughly with tap water. Exfoliated buccal cells were obtained by gently scraping the inside of both cheeks with a moistened wooden spatula. Cells were smeared on to a clear glass slide and fixed with 95% ethanol for a minimum of 15mts. A minimum of two smears from each individual was taken so that at least 100 cells per smear were obtained. Smears thus obtained were stained with papanicolou. The smears were examined under a light microscope; changes within the cytoplasm and nucleus were noted, frequency of micronuclei was noted using the "Tolbert's criteria". A cytomorphometric analysis of the buccal cells was done using leica 1000 software.

RESULTS

A total of 60 cases of oral submucous fibrosis cases were identified and included in the study.

Pattern Of Consumption Of Tobacco Use Among Individuals With Oral Lesions

60% of the subjects were addicted to gutkha (pan masala with tobacco) 20% of the subjects consumed pan masala without tobacco I4% of the subjects used betel quid (a preparation with areca, lime and catechu) 4% have used tobacco in its leafy form or as dentifrice (gulmanjan) 2% cases used tobacco but did not reveal its pattern of usage

Cytomorphometric Analysis Of Buccal Cells In Submucous Fibrosis Compared With Healthy Controls

Mean cellular diameter of cells affected by OSMF was 24.97µm with a standard deviation of 3.129. The mean cellular diameter in exfoliated buccal cells of healthy controls was 49.51µm with a standard deviation of 7.308µm.

The mean nuclear diameter in exfoliated buccal cells affected by OSMF was 6.19µm with a standard deviation of 1.219, while in normal/healthy controls it was found to be 8.83µm with a standard deviation of1.096µm. A bi-group comparison using PosthocDunnett test was done andthe mean difference in the cell diameter of cells with OSMF as compared withnormal was found to be lesion group 24.53µm and according to Dunnett test this difference was highly significant ($p<0.001$).

The mean difference in nuclear diameter of cells with oral lesions as compared with healthy controls was found to be 2.64µm and according to Dunnett test this difference was highly significant ($p<0.001$).

Frequency Of Micronuclei In Submucous Fibrosis Compared With Healthy Controls

The frequency of micronuclei per cell in individuals with OSMF was found to be 2.37 with a standard deviation of 1.100 while in healthy controls the frequency of micronuclei per cell was 1.02 with a standard deviation of 0.139.

Clinical Grades And Related Histopathology

Clinically the individuals were identified on the basis of their clinical symptoms and the mouth opening achieved. Clinically four grades of oral submucous fibrosis were identified and were correlated with their histopathology

Clinical stage I and related histopathololgy

A burning sensation was experienced by the participant on consuming hot and spicy food. There was a blanching

of the buccal mucosa with no palpable fibrous bands. Histologically epithelium showed hyperkeratosis, a fine fibrillar collagen network was present, presence of young fibroblasts, blood vessels were normal, dilated or congested. Inflammatory were cells present.

Clinical stage II and related histopathology

Mouth opening is reducedwith severe burning sensation, blanching of faucial pillars, soft palate, buccal mucosa and labial mucosa. Palpable fibrous bands were found in the buccal mucosa. The epithelium undergoesatrophy, rete ridges were less prominent with a variable degree of keratinization. The juxta epithelial layer showed early hyalinization. Thickened collagen bands were present and fibroblasts were seen in moderate number. Blood vessels were dilated and congested.(figure 1)

Fig 1: Shows 75% Mouth Opening, Epithelium Undergoes Atrophy

Clinical stage III and related histopathology

Mouth opening was reduced by 50%. Severe burning sensation was present even in the absence of stimulation. Blanching was seen in the faucial pillars, soft palate, buccal mucosa and labial mucosa. Palpable fibrous bands with unilateral/ bilateral lymphadenopathy were present.Epithelium undergoes atrophy, rete ridges are less prominent, moderate hyalinization. (figure 2)

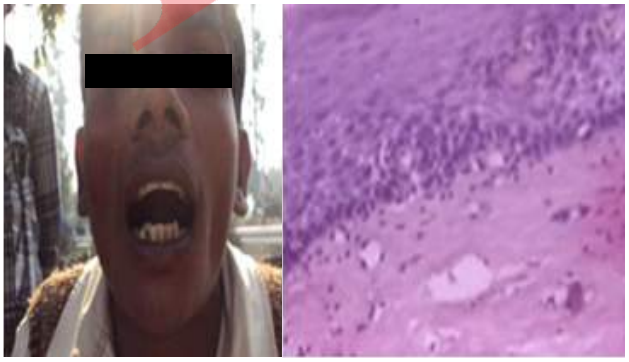

Fig 2: Shows A 50% Mouth Opening, Rete Ridges Are Less Prominent

Clinical stage IV and related histopathology

Mouth opening was less than 25%. Hyposalivation was present, unilateral/bilateral lymphadenopathy was present. Intraoral inspection was not possible as there was very little mouth opening.

Atrophic epithelium was present; there was an absence of rete ridges/pegs. Blood vessels were completely obliterated. There was an extensive degeneration of muscle fibres. (figure 3)

Fig 3: Shows Less Than 25% Mouth Opening And There Is An Absence Of Rete Pegs

Cytological Grading of OSMF

The results obtained after a cytomorphometric analysis of exfoliated buccal cells of healthy individuals (using Leica sotware), analysing the frequency of micronuclei (using Tolbert,s criteria) and observing the degree of keratinization in cells by observing colour change of papanicolou stain, the data was compiled to achieve a cytological grading of oral submucous fibrosis. The results thus compiled indicate changes within the buccal mucosa even in the absence of any visible clinical symptoms.

Grade I We observed 2-3 micronuclei, with a magenta hue to the cytoplasm, an altered shape of the cell was seen with a cellular diameter of 27.3µm and a nuclear diameter 6.1µm. (figure 4)

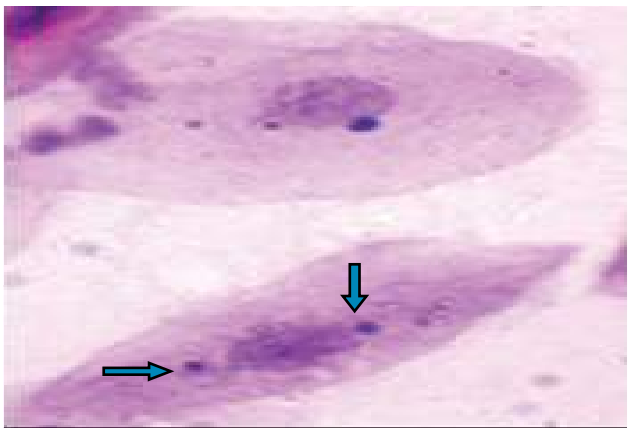

Fig 4: Shows Altered Shape Of Cell With 2 Micronuclei, Magenta Hue In The Cytoplasm Present

Grade II We observed 3-4 micronuclei, there was an altered shape of the cell, the cytoplasmic hue was purplish, the cellular diameter was 26.52µm and the nuclear diameter was 5.92µm (figure 5).

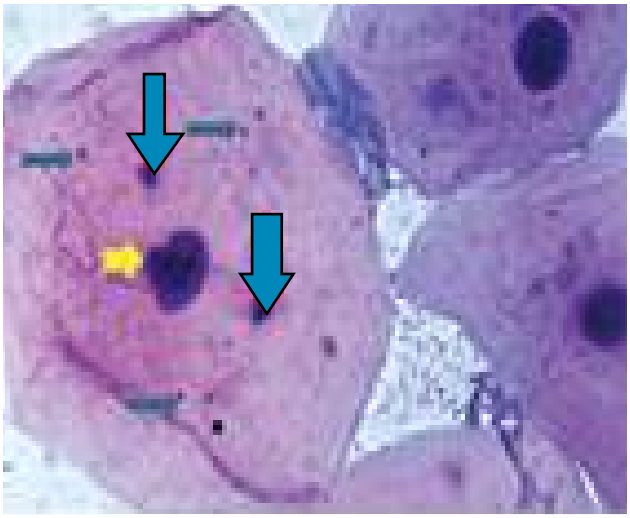

Fig 5: Shows A Purplish Hue In The Cytoplasm, 3 Micronuclei Present

Grade III We observed 3-4 micronuclei, there was a purple hue in the cytoplasm, the cellular diameter decreased considerably to 25.35µm and the nuclear diameter was 5.5µm (figure 6).

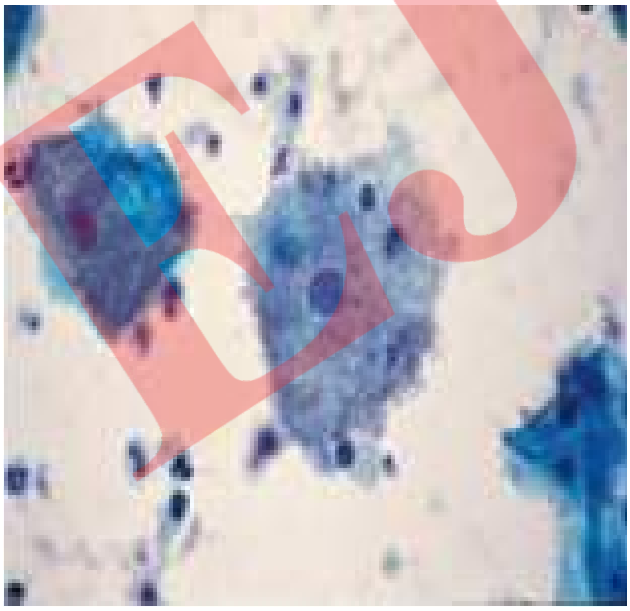

Fig 6 Shows A Purplish Hue To The Cytoplasm With 3-4 Micronuclei

Grade IV We observed plenty of keratinised cells, there was a green hue to the cytoplasm, and many pyknotic cells were seen there was a marked reduction in the cytoplasmic diameter (figure 7).

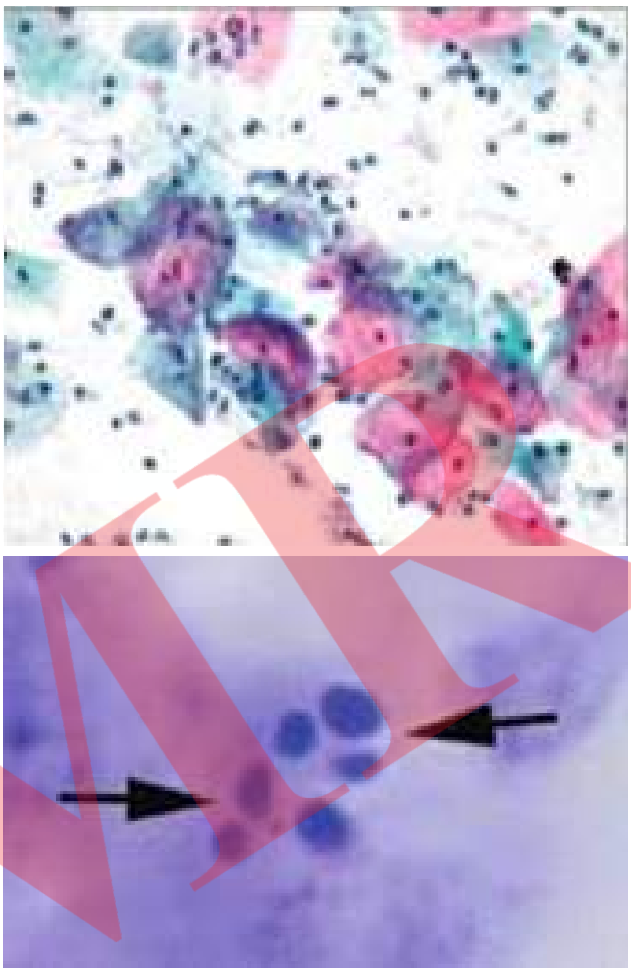

Fig 7: Shows Numerous Keratinized Cells With Pyknotic Nuclei

DISCUSSION

Cellular Diameter

We found that the cellular diameter in the cells of the control group was the highest while it was low in participants with oral lesions. The cellular diameter of participants with submucous fibrosis in our study was 24.97µm while there were other studies on premalignant lesions such as leukoplakia andaccording to Hegde V et al, Hande AK et al and Ramesh T et al[14,15,16] the cellular diameter was 29.71+/- 5.73µm, 57.75+/-4.66µm and 41.32+/-0.13µm respectively.

Nuclear Diameter

In a study by Hegde V[14] et al the nuclear diameter in leukoplakia was 6.317µm, in a study by Hande AK et al[15] it was found to be 9.12µmin cells with oral lesion while it was 9.04µm in a study on oral lesions by Ramesh T et al [16]. In our study we found the diameter of the nucleus in the cells affected by submucous fibrosis to be 6.19µm.

Nuclear Cytoplasmic Ratio

Cowpe JG et al reported that the change in the nucleocytoplasmic ratio is because of the change in the diameter of the nucleus as well as the cytoplasm [17]. Franklin CD[18] and Smith CJ 1980[19] reported that a calculation of the nucleo-cytoplasmic ratio can be related to a change in the nuclear and cytoplasmic volume thus predicting accurate morphological changes of the cell.

Micronuclei And Oral Lesions

In oral submucous fibrosis patients arecanut chewing leads to an increase in the total serum protein and the levels of ascorbate and iron are usually found to be lower than the normal value. The collagen content is increased significantly in oral submucous fibrosis patients in the third stage of the disease. When smokeless tobacco is consumed along with its smoked form, the chances of an increase of the grade of submucous fibrosisis more due to the other reactive agents present in cigarettes, these carcinogenic agents are responsible for the DNA damage. Chromosomal aberrations and micronuclei serve as excellent markers for assessing the extent of DNA damage.

According to a study byParvathi Devi et al in the year 2011[20]there was a progressive increase in the number of micronucleated cells while observing normal, precancerous and cancerous cells exfoliated from the buccal mucosa. She observed that in normal cells the frequency was 0.06%, in precancerous lesions it was 0.12% and in cancerous lesions it was 0.45%, thus indicating cytogenetic damage to the epithelium. Haldar et al 2004[21] found the frequency of micronuclei in precancerous to be 0.63% and in cancerous lesions it was 1.36%. Palve DH et al 2008[22]concluded that there was a gradual increase of micronuclei from normal cells which was 0.21% to cancerous lesions which was 1.84%. An observation of micronuclei in normal, precancerous and cancerous epithelial cells by Casartelli et al in 2000[23]revealed that the frequency of micronuclei in malignant lesions was more when compared with normal buccal mucosal cells hence this trend indicates neoplastic progression.

Himadri Kalita et al in the year 2013 [24]observed buccal cells of betel quid chewers and found that the micronuclei count was less in females (4.2+/-0.96)as compared to males (6.6+/-1.95). SmitaJyoti et al in 2013[25] conducted her study on 25 oral submucous fibrosis patients using a special stain acridine orange. She found an increase in the frequency of micronuclei in the buccal cells of gutkha chewers as compared to participants with no such habit.In our study the frequency of micronuclei per cell in participants with oral lesions was 2.37 with a standard deviation of 1.100

Pattern Of Consumption Of Tobacco In Oral Submucous Fibrosis

Oral submucous fibrosis is an insidious disease and comes under the category of potentially malignant disorder. Reichart et al 2006[26]and Kumar KK et al 2007)[27] suggested that though oral submucous fibrosis is a major health hazard in India but is also spreading to other countries as a result of transmigration of population. Thirty three submucous fibrosis patients were a part of our study and the ratio of male participants was more than the female participants. There was a 5:1 male to female ratio in a study conducted by Hazarey et al [28]from Nagpur in the year 2005. According to Zhang et al who conducted his study in china in the year 2006[29]the prevalence of betel quid chewing was 64.5%-82.7% in the province of Hunan and Hainan. 0.9%-4.7% of the population was affected by Oral submucous fibrosis of which the age group most affected bythis disease was between 30-49 years. Our study showed similar results where the prevalence of OSMF was high. In our study, 80% of the patients, chewed paan masala with tobacco (gutkha) 20% pan masala without tobacco, 14% of the patients used betel quid. In a study by Kumar et al[27]in Chennai nearly 81% of the participants indulged in the habit of chewing areca nut either raw or in commercially prepared forms. Hazarey et al[28]conducted a study in Nagpur (Western India) and found the consumption pattern of smokeless tobacco was different in both men and women. Women consumed more of areca nut while men preferred mawa or khara. Babu et al 1996[30] reported that chewing of pan masala/gutkha is responsible for causing oral submucous fibrosis rather than betel quid. Thomas et al in 2003[31] conducted a study in South India and suggested tobacco chewing was one of the major factors causing oral cancer. In our study only 2% males were habituated to alcohol, but no consistent correlation was found between the OSMF and smoking/alcohol consumption.Ho et al [32] conducted a studyin the year 2007 and found that a combination of alcohol consumption along with smokeless tobacco was more injurious causing malignant transformation of oral submucous fibrosis. In his study 10.4% of the patients consumed smokeless tobacco and were addicted to smoking and alcohol also. Similarly, Auluck et al [33]in his study from Canada found that smokeless tobacco used with its smoked form along with alcohol consumption causes leukoplakia, oral submucous fibrosisand verrucous lesions as well.

Clinical Grading Of Oral Submucous Fibrosis

Clinically, trismus (limited mouth opening) is an important symptom of oral submucous fibrosis. In the

current study 3 out of the 33 cases had a 75% mouth opening, 13 out of 33 cases had a 50% mouth opening while 17 cases had a mouth opening which was either 25% or less. Kumar et al [26] also reported that 75% males and 80% females with oral submucous fibrosis patients had stage II disease and suggested that this could be due to the fact that the majority of the patients reported for treatment only after their inability to open their mouths ignoring the early signs of the disease. Hazarey et al[29] also reported that most of the patients with oral submucous fibrosisin their study, had stage III trismus.

CONCLUSION

Use of tobacco in smoked and smokeless forms is one of the prime factors responsible for precancerous and cancerous lesions. A relative lack of awareness regarding harmful effects of tobacco is a major reason for the same. There is a need to accumulate data over a large geographical area which will thereby aid in formulating appropriate prevention and control measures. Our study thus points out the importance of early recognition of early changes occurring within a cell even when the mucosa is not showing any visible signs of an insidious disease. A quantitative assessment of smears obtained through exfoliative cytology improves the diagnosis thus aiding in better treatment and prognosis of a disease. Certain available biomarkers can help in the screening of particular premalignant lesions, showing a trend from a premalignant lesion to a neoplastic lesion but they will not be able to state with certainty whether or when a malignant change may happen. Biological monitoring cannot replace the medical check-up and histopathological diagnosis when cancer is suspected. However, it is useful for prescreening programs of high-risk groups, especially for those cancers such as squamous cell carcinoma.

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