Study of Nuclear Anomalies and Cytological Features in Buccal Mucosa of Tobacco Users

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ABSTRACT

Introduction: Tobacco, a chemical carcinogen, has genotoxic effects on buccal mucosal cells. The present study is performed to evaluate all cytological features in exfoliated buccal mucosal cells of tobacco users and to compare them in different form of tobacco users i.e. smokers and smokeless "chewers". Also to evaluate whether Micronuclei frequency can be used as a biomarker and screening tool to assess genotoxicity in tobacco users.

Material and methods: Oral buccal mucosal exfoliated cells from total 80 tobacco users-40 smokers and 40 smokeless tobacco chewers, and 20 contols with healthy mucosa were taken. Micronuclei frequency and other nuclear anomalies were compared using Papanicolaou stain. The results are analyzed statistically using t-test and Fisher's Exact test.

Results: Smears of individual with tobacco habits showed significant increase in Mean Micronuclei (MN) per 100 screened cells (p=0.01). Cytological features like orangophilia, keratinisation, degenerative changes and all nuclear anomalies are increased in tobacco users but only nuclear fragmentation show statistically higher incidence. No statistical significant difference was found for Mean MN and other nuclear anomalies between tobacco smokers and smokeless tobacco "chewers".

Conclusion: Nuclear anomalies and Mean MN frequency are increased in any form of tobacco users, thus MN frequency can be used as a biomarker and a screening tool for genotoxicity in any form of tobacco users.

Keywords: Cytology, Micronuclei, Nuclear Anomalies, PAP, Tobacco Smoker.

INTRODUCTION

Tobacco is chemical carcinogen, having genotoxic effects. Buccal cells are the first barrier for the inhalation or ingestion route and are capable of metabolizing proximal carcinogen to reactive products, result in genomic instability and in late stage reflected grossly as submucous fibrosis, leukoplakia, erythroplakia and finally in squamous cell carcinoma. Microscopic changes are said to occur earlier in buccal mucosa which include micronuclei, and other nuclear anomalies like karyorrhexis, karyolysis, pyknosis, binucleation, fragmentation and broken egg nuclei.[1]

Micronuclei (MN) are small, extra-nuclear bodies which have separated from the main fragment, generated during cellular division by late chromosomal fragments because of their association with chromosomal aberrations. They have generated interest as a cytological feature of genotoxicity.[2,3,4,5] The MN test is a quantitative measure of the genotoxic action of carcinogens and mutagens.[6,7] MN scoring can

be used as a biomarker to identify different pre-neoplastic conditions much earlier than the manifestations of clinical features.[2,3,4,5,6,7,8] Few studies have been done MNs in tobacco smokers but only a few on tobacco chewers.

Large number of tobacco chewers (mava/ gutka) suffer from high morbidity and mortality due to oral cancers. Since no screening tests are available to show morphological changes in buccal mucosa amongst these mava and gutka users, this study was undertaken to assess cytological assessment of buccal mucosa at Tertiary Medical Care Centre with the following aims:

To evaluate the cytological features, Micronuclei (MN) frequency and other Nuclear anomalies (NA) in buccal mucosal cells of tobacco users,

To compare the micronuclei (MN) frequency and other Nuclear anomalies in different form of tobacco use- smoking and smokeless tobacco("chewers")

To access if MN frequency can be used to access the genotoxicity in tobacco users.

MATERIAL AND METHODS

A prospective comparative study was conducted over a four month period in a tertiary care hospital. To avoid confounding factors found in other studies like age, sex and alcohol, only non alcoholic male between 25-45 years were assessed. The subjects were selected depending on the following criteria:

Inclusion criteria

Group 1:   Controls: 25-45 years male with no habit of smoking or tobacco chewing, no buccal mucosal lesions – sample size 20

Group 2:   Cases: 25-45 years male Tobacco users - sample size 80
2a) Smokers- (5-20 bidi/cigarette smoking/day for more than 5 years)
2b) Smokeless tobacco "chewers"- (2 or more mava/day for more than 5 years)

Exclusion criteria

1.   Patients who had oral x-ray in previous one month.
2.   Patients who had received treatment for the buccal

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	Control (n=17) Mean (± 1 SD)	Cases (n=71) Mean (± 1 SD)	Unpaired t-test (P value)	Chewers (n=33) Mean (±1SD)	Smokers (n=38) Mean (± 1 SD)	Unpaired t-test (P value)
MN/100 cells	20.1 (± 17.6)	31.1 (± 29.9)	0.14	18.4 (±11.3)	19.3 (±10.4)	0.72
MNt /100 cells	11.7 (± 8.2)	14.0 (± 11.1)	0.43	12.4 (±8.5)	15.3 (±12.9)	0.27
Mean MN	1.5 (± 0.4)	2.2 (± 1.0)	0.01 Statistically Significant	2.1 (±1.0)	2.2 (±0.9)	0.69
Table-R1: Comparison of MN, MNt and Mean MN per 100 screened cells.						

Frequency of Micronucleated cells	Controls (n=17) Mean (± 1 SD)	Cases (n=71) Mean (± 1 SD)	Unpaired t-test (P value)
1 MNt /100 cells	7.0(±4.2)	6.7(±5.1)	0.83
2 MNt /100 cells	2.6(±2.2)	3.5(±4.0)	0.38
3 MNt /100 cells	1.3(±1.7)	2.1(±3.1)	0.32
4 MNt/100 cells	0.5(±0.9)	1.1(±1.6)	0.14
≥ 5 MNt/100 cells	0.2(±0.6)	0.7(±1.1)	0.12
Table-R2: Frequency of 1,2,3,4, ≥ 5 micronucleated cells among controls and cases.			

	Controls n=17	Cases n=71	Fisher's Exact test
Condensed nuclei	2	6	P=0.65
Karyorrhexis	10	26	P=0.11
Karyolysis	1	13	P=0.29
Binucleated cells (BN)	0	4	P=1.00
Broken egg nucleus (BEN)	0	7	P=0.34
Nuclear budding (NB)	2	2	P=0.17
Fragmented nuclei (FN)	2	29	P=0.03 Statistically significant
Table-R3: Nuclear anomalies other than MN among controls and cases.			

number of micronucleated cells (MNt) and mean micronuclei frequency (Mean MN= Total MN/MNt) per 100 screened cells were counted.

The criterion which was developed by Tolbert et al.[2,9] were used for counting the micronuclei. Parameters for the cells to be scored are:

1. Intact cytoplasm and relatively flat cell position on the slide,
2. Little or no overlap with adjacent cells,
3. Little or no debris, and
4. Nucleus normal and intact, nuclear perimeter smooth and distinct.

To designate an extra nuclear body as a MN, the following criteria given by Tolbert *et al.*[2,9] were used:

1. Rounded smooth perimeter suggestive of a membrane
2. Less than a third the diameter of the associated nucleus, but large enough to discern shape and color
3. Staining intensity similar to that of the nucleus
4. Texture similar to that of nucleus
5. Same focal plane as nucleus
6. Absence of overlap with or bridge to the nucleus.

Nuclear Anomalies (NA) other than MN like Condensed Nuclei, Karyorrhexis, Karyolysis, Binucelation, Broken egg nuclei and fragmented nuclei were also counted in both study groups.

STATISTICAL ANALYSIS

"Unpaired t-test" was used for the comparison of MN, MNt, and Mean MN among tobacco users (cases) and tobacco non-users (controls) and also used for comparison between two different form of tobacco users- smokers and smokeless tobacco -chewers. For comparison of different types of meta-nucleated cells among cases and controls, "Fisher's Exact Test" was used. All the Statistical analysis was done by using 'Graphpad online' software. P<0.05 was considered as statistically significant in all results.

RESULTS

A total of 80 cases and 20 controls were studied. Out of 80 cases, 40 were tobacco chewers and 40 were smokers. Three control and nine cases did not have adequate cellularity, so 17 controls and 71 cases were analysed statistically. Table R1,R2,R3.

Mean MN showed significant high value in cases compared to controls (p= 0.01) (Table R1).

mucosal lesions like radiotherapy and/or chemotherapy for the oral lesions.

3. Chronic alcohol consumer.
4. Patients suffering from blood dyscrasias and diabetes mellitus.
5. Female tobacco users.

Method

The subjects were asked to rinse their mouth thoroughly before taking the scrapings in order to remove food particles, debris and oral bacteria from the oral cavity. Using a dry wooden spatula, the scrapping was taken from right buccal mucosa and spread over a clean glass slide in circular manner from central of slide to periphery. The smears were wet fixed, in methyl alcohol for 30 minutes and stained with Papanicolaou-ready to use stain. Slides were examined under scanner for cellularity and oil for nuclear morphology. Over all cytological features were noted. 100 cells were screened from each slide in zig-zag method to avoid repeatation of count. Total Micronuclei (MN), total

Control had 20 MN in 12 MNt cell/ 100 cells where as tobacco users had 31 MN in 14 MNt cell/100 cells. The range of MN and MNt cells in both groups was very wide. Smokers and chewers both have almost similar MN, MNt and Mean MN values.

The mean of 2, 3, 4 and ≥5 micronucleated cells especially of 4 and ≥5 are higher in tobacco users. However the difference was not of statistical significance(Table R2).

Frequency of all nuclear anomalies is increased in tobacco users but only fragmented nuclei show statistical higher incidence (Table R3).

DISCUSSION

Many studies have been done in India and abroad on MN frequency in 2 or more of the groups of non tobacco users, users with premalignant lesion or carcinoma. Most have assessed only smokers.[3,10,11,12,13] In this study overall cytological findings in buccal mucosa of tobacco users was studied and compared with controls. We also compared MN amongst chewers and smokers of tobacco, evaluated the number of cells with more than 1 MN and nuclear anomalies other than MN using a PAP stains rather than using Giemsa which has been done in most previous studies.[12,14,15] In PAP, smear is wet fixed in alcohol which gives a clear background, whereas in giemsa stain, the smear is air dried and stained smear has background which is full of debris and salivary proteins, thus masking the counting of MN.

Control and cases selected for the study were all nonalcoholic males who were 25-45 years. This was to remove confounding factor of alcohol consumption, age and gender noted in other studies.[12,14,15]

Cytology of buccal mucosa in Controls

The buccal mucosa of controls exfoliated easily and smeared in a monolayer (figure 1a). Squamous cells showed minimal overlapping and folding. They did not show any orangophilia, keratinisation and necrotic changes. Nuclear karyorrhexis and proportionate karyolysis were seen (figure 1b,1c). MN were also seen in many of the controls. However BN, BEN and fragmentation like nuclear anomalies were not seen.

Cytology of buccal mucosa in Cases

The ease of exfoliation decreased in tobacco users. Squamous cells did not spread well in PML. Figure 2a,2b shows buccal mucosal scrapping in a case of sub mucous fibrosis and erythroplakia respectively. There was increased orangophilia and folding of cells, keratinisation and degenerative changes in the cells. Large no. of cases showed mucosal cell contamination with bacteria. These could be easily distinguished from MN (figure 2c). It indicates that oral hygiene was poor in tobacco users.

Nuclear changes (Figure 3) seen in tobacco users were MN, fragmentation, karyorrhexis, karyolysis, broken egg nuclei, condensed nuclei, and binucleation in decreasing order of frequency (71/29/26/13/7/6/4 out of 71 cases respectively).

Two bidi smoker without any oral lesion showed CIN 3 (figure 4a) and malignant cells (figure 4b) respectively.

Fig 1a)Squamous cells in controls. PAP, 10x   Fig 1b) Karyorrhexis and Karyolysis in controls, PAP, 100x

Fig 2 Buccal scrapping in a) erythroplakia and b) submucous fibrosis, PAP, 10x. Both show marked overlapping and orangophilia of cells.   Fig 2c) Bacterial Contamination, PAP,100x

Squamous pearl formation was also seen in the later case (figure 4c).

The result of the present study is comparable with other studies (Table D1) that have showed smoking causes an increase in mean number of micronucleus indicating that tobacco has genotoxic effect.

The wide range of MN and MNt cells in controls and cases seen in table R1 suggest that multiple confounding factors exist in both groups which deflect the expected results. The probable factors in the population of this study could be anaemia, dietary vitamin deficiencies, environmental pollution- exposure to organic solvents, diesel derivatives, polycyclic aromatic hydrocarbons, lead containing paints, remnants of pesticides in agricultural products and solvents and arsenic contaminated drinking water.

Micronucleus frequencies would vary significantly between samples. Such pattern would also be produced if large differences occurred in the dosage of the carcinogen in repeated exposures. As micronuclei tend to decrease in frequency with time as chromosomal damage leads to cell

Study name	n = control/ cases	Stain	Mean MN in control	Mean MN in cases	Remark
Ozkul et al.[25] 1997	15/39	Feulgen	-	1.99	Significant
Konopacka et al.[26] 2003	70/50	Feulgen	0.55	1.50	Significant
Naderi et al.[28] 2012	23/40	Feulgen	0.94	1.95	Significant
Grover et al.[27] 2012	15/45	Feulgen	1.60	3.80	Significant
	15/45	PAP	7.70	16.80	Significant
Kamath et al.[3] 2014	50/50	PAP	34.92	57.96	Significant
Our study	20/80	PAP	1.53	2.19	Significant
Table-D1: Comparison of MN in smokers (cases) and non-smokers (controls)					
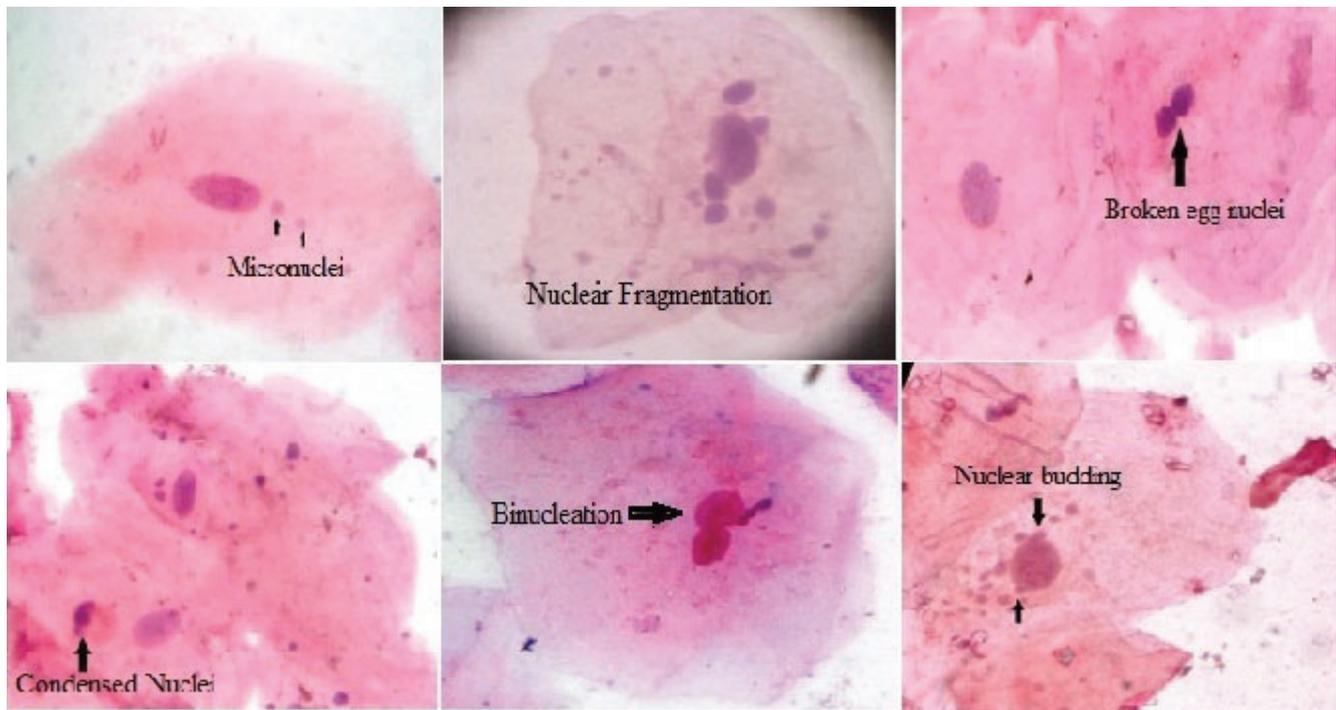
Fig 3 Nuclear anomalies in tobacco users, PAP, 100x
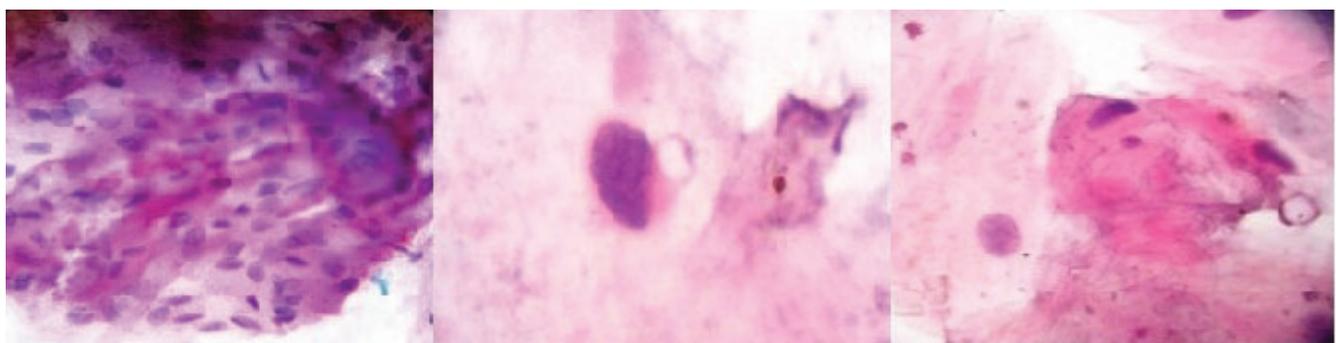
Fig 4a) CIN 3, PAP, 100x   Fig 4b) Malignant cell, PAP, 100x   Fig 4c) Squamous pearl, PAP, 100x

Study name	n = chewer/smoker	Stain	Mean MN in Chewer	Mean MN in Smoker	Remark
Palaskar et al.[12] 2010	15/15	PAP	45	22.07	Significant
Our study (Table R1)	40/40	PAP	2.14	2.23	NS
Table-D2: Comparison of MN in tobacco chewers and smokers.					

death or micronuclei are lost during cell division. It seems likely that cells with more chromosomal damage, and hence more micronuclei, would be lost at a higher frequency than those with less damage.

Many of the studies have reported increases in MNs in the buccal mucosa cells, have been performed in subgroups of subjects with specific lifestyle habits, i.e. chewers of betel quids (areca nut, betel leaves, slaked lime and tobacco); reverse smokers from India and Philippines; snuff dippers from Canada and users of Khaini tobacco from India (tobacco mixed with slaked lime).[13-15]

Comparative studies on individuals who consumed tobacco in different forms are scarce.[17] One such available study is compared in table D2.

Palaskar et al.[17] reported values of MN in 500 cases hence their figure appear to be very high. They found that smokers have lesser value than chewers. In our study no significant difference was found. It may be due to two reasons. The nicotine content of Indian brands of smoking tobacco (e.g. in bidies) is higher as compared to international brands (e.g. in cigarettes and cigars). And in smokeless tobacco "chewing form", the nicotine content is much lower than the smoking form, but the average daily consumption and its direct contact with the oral buccal mucosa has made it comparable to smoking form.

To find whether tobacco induces nuclear anomalies, other than micronuclei, we have evaluated the occurrence of different types of metanucleated cells in both controls and cases. No studies are available for the discussion of this. Cells with 1 MN are highest in both cases and controls, but the percentage of >1 MN increases in tobacco users.

Directly comparable studies are not available for the discussion of different nuclear anomalies. Nersesyan et al.[15] showed condensed chromatin, karyorrhexis, karyolysis and binucleation were 54%, 146%, 350% and 117% higher in smokers than in non-smokers. In our study, though fragmentation, karyorrhexis, karyolysis, broken egg nuclei, condensed nuclei, and binucleation have increased frequency, only fragmentation is significantly increased.

Condensed nuclei, karyorrhexis, karyolysis, pyknosis are also seen in cells undergoing necrosis and cannot be regarded as reliable markers for increased DNA damage and cancer risk. Nuclear anomalies reflect the consequences of any form of cell injury. However, micronuclei (chromosomal breakage or loss), nuclear budding (gene amplification), broken egg nuclei (elimination of amplified gene) and fragmented nuclei (mitotic failure) do not occur as part of normal cell death or in apoptosis. Hence these anomalies indicate some sort of DNA damage. BN being a result of defective cytokinesis cannot be ideally expected in tobacco injury. It was seen only in 4 cases in our study compared to 117% increased in the study of Nersesyan et al[15].

In literature, a large number of discrepancies are seen in the numerous studies on MN. These may be due to variables like age, gender, diet, lifestyle habits and oral hygiene, methodological factors which includes differences in cell collection, fixation and staining techniques, selection of cells for scoring, total number of samples studied, scoring criteria used for MN and other nuclear anomalies. It may also be due to difference in the tobacco form evaluated (smoke vs. smokeless agents) and difference in tobacco content of cigarette / bidi and mava / gutka among different brands.

CONCLUSION

The buccal mucosa in tobacco users especially in those with oral lesions exfoliate with difficulty and show increased keratinisation, overlapping and degenerative changes. The nuclear anomalies like fragmentation, karyorrhexis, karyolysis, broken egg nuclei, condensed nuclei, and binucleation are also seen in tobacco users with increased frequency compared to controls, but only fragmentation of nuclei is seen significantly higher in tobacco users. Mean Micronuclei was frequency significantly higher in tobacco users. Thus micronuclei frequency can be used to access genotoxicity and as a screening tool. The Micronuclei frequency in tobacco chewers and smokers was nearly equal, but no difference was noted in the different types of tobacco user.

ACKNOWLEDGEMENTS

ABBREVIATION

MN -Micronuclei, MNt- Micronucleated cell, NA-Nuclear Anomalies, BN- Binucleated, BEN- Broken Egg Nuclei, NB- Nuclear Budding, FN-Fragmented Nuclei, PAP-Papanicolaou stain, PML- Pre-malignant Lesion

REFERENCES

1. Standring S, Ellis H, Healy JC, Jonson D, Williams A, Collins P et al. Grays Anatomy: The anatomical basis of clinical practice. 39th ed. Elsevier, Churchill Livingstone: 2006, p 581-83.
2. Holland N, Bolognesi C, Kirsch-Volders M et al. The micronucleus assay in human buccal cells as a tool for biomonitoring DNA damage: the HUMN project perspective on current status and knowledge gaps. Mutation Research 2008; 659: 93-108.
3. Kamath VV, Anigol P, Krishnan and Setlur. Micronuclei as prognostic indicators in oral cytological smears: a comparison between smokers and non-smokers. Clinical Cancer Investigation Journal. 2014; 3: 49-54.
4. Dhingra PL-Diseases Of Ear, Nose And Throat.3rd ed. Elsevier Publications: 2004, p 265-67, 270-76.
5. Shaefer WG, Hine MK, Levy BM. Text book of oral

pathology. 4th ed. Harcourt Brace and Company Asia Pte Ltd.: 2000, p 110,113-27.

6. Bennel SE, Lippman SM., Michael J, Walgovich J, Marcovelasco J, Martin JW et al. Micronuclei a biomarker for chemoprevention trials. Results of a randomized study in oral pre-malignancy 2008;41: 713-792.

7. Pawitan JA. Biological tests for mutagenesis in man: a clinical review. Medical progress 1999; 16-18.

8. Genetic Toxicity. Available From (Http://Www.Cerep. Fr/Cerep/Users Pages/Download //Documents / Marketing / Pharmacology%20 Notesgenotoxicity.Pdf.) Jan 2007.

9. Rosin MP. The use of micronucleus test on exfoliated cells to identify anti-clastogenic action in humans: a biological marker for the efficacy of chemopreventive agents. Mutation Research. 1992; 267: 265-276.

10. Tolbert PE, Shy CM, Allen JW. Micronuclei and other anomalies in buccal smears: a field test in snuff users. American Journal of Epidemiology 1991; 134: 840-50.

11. Konopaka M. Effect of smoking and ageing on micronucleus frequencies in human exfoliated buccal cells. Neoplasma 2003; 50: 380-2.

12. S Grover, ABR Mujib, A Jahagirdar, N Telagi and PG Kulkarni.A comparative study for selectivity of micronuclei in oral exfoliated epithelial cells.J Cytol. 2012; 29: 230-235.

13. Naderi NJ, Farhadi S, Sarshar S. Micronucleus assay of buccal mucosa cells in smokers with the history of smoking less and more than 10 years. Indian Journal of Pathology And Microbiology. 2012; 55: 433-438.

14. Dindgire SL, Gosavi S, Kumawat RM, Ganvir S, Hazarey V. Comparative study of exfoliated oral mucosal cell micronucleus frequency in potentially malignant and malignant lesions. International Journal of Oral and Maxillofacial Pathology. 2012; 3: 15-20.

15. Nersesyan A, Kundi M, Atefie K, Hermann RS and Knasmuller S. Effect of staining procedures on the results of micronucleus assays with exfoliated oral mucosa cells. Cancer Epidemiol Biomarkers Prev. 2006; 15:1835-40.

16. Ozkul Y, Donmez H, Erenmemisoglu A, Demirtas H, Imamoglu N. Induction of micronuclei in smokeless tobacco on buccal mucosa cells of habitual users. Mutagenesis 1997:12; 285-7.

17. Palaskar S And Jindal C. Evaluation of micronuclei using Papanicolaou and May Grunwald Giemsa stain in individuals with different tobacco habits – a comparative study. Journal of Clinical and Diagnostic Research 2010;4: 3607-3613.

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