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ORIGINAL ARTICLE

Evaluation Of Micronuclei Using Papanicolaou And May Grunwald Giemsa Stain In Individuals With Different Tobacco Habits – A Comparative Study

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ABSTRACT

Aims: To compare the Papanicolaou's (Pap) and May Grunwald's Giemsa (MGG) staining techniques which are done to detect micronuclei (MN) in exfoliated buccal mucosal cells in individuals with different tobacco habits. To determine the severity of human buccal cell changes which are associated with smoking and smokeless (''chewing'') tobacco (SLT).

Methods and Material: A total of 45 male subjects (15 smokers, 15 smokeless tobacco users and 15 non users/ non smokers) were examined. Two cytological smears were taken from the apparently normal buccal mucosa from each individual. 45 smears (1 per individual) were wet fixed and stained with Pap and the remaining 45 smears were air dried and stained with the MGG stain. All the smears were assessed for cellularity and were scored for MN. **Statistical analysis used:** The ANOVA (one way analysis of variance) was used to analyse the frequency of cells with micronuclei. Bonferroni multiple comparisons were done to determine the significance ($p<0.05$) of the mean difference.

Results: MNs were easily seen in the clear cytoplasm in the Pap smears. Regarding the MGG smears, bacteria and cell debris masked the effect of the MNs as compared to the Pap smears, where the fixative destroyed the bacteria and clearly demarcated the cell boundaries. The score of the MN frequency decreased as we moved from the smokeless tobacco chewers to the smokers and then to the non users and the non smokers. **Conclusions:** Pap is a better stain as compared to MGG for counting micronuclei. Smokeless tobacco chewers showed an increased number of MNs as compared to the smokers, thus laying emphasis on the greater carcinogenic potential of tobacco which was used in the chewable form.

Key-words: Micronuclei, Smokers, Smokeless tobacco users, Papanicolaou, May Grunwald's Giemsa

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Introduction

The buccal cell micronucleus (MN) assay was first proposed in 1983 [1] and it continues to gain popularity as a biomarker of genetic damage in numerous applications. MN assays provide information on the cytogenetic damage in the tissues, that are targets of human carcinogens and from which carcinomas can develop. Oral squamous cell carcinomas are characterized by complex karyotypes that involve many chromosomal deletions, translocations and structural abnormalities. Cells from these type of tumours often have errors in chromosome segregation that lead to the formation of a lagging chromosome or chromosome parts that become lost during the anaphase stage of cell separation and are excluded from the reforming nuclei. The laggards are observed in the cytoplasm as micronuclei [2],[3].

Significantly higher frequencies of MNs have been observed in exfoliated buccal cells, from

people who are exposed to organic solvents, antineoplastic agents, diesel derivatives, polycyclic aromatic hydrocarbons, lead-containing paints and solvents and drinking water which is contaminated with arsenic [4]-[23]. Recent studies have also suggested the genotoxicity and the cytotoxicity of the urban air pollution and ozone during the summer season, particularly in places with high ambient levels [24]-[26].

The lifestyle factors that are associated with genetic damage include smoking, alcohol consumption and diet, especially vitamin deficiencies and supplementation [2],[27]. A majority of the studies which reported a significant increase in MNs in the buccal mucosa cells, which were related to a risk of oral cancer, were performed in subgroups of subjects with specific lifestyle habits, i.e. chewers of betel quids (areca nut, betel leaves, slaked lime and tobacco) from India, Taiwan and Philippines; reverse smokers from India and Philippines; snuff dippers from Canada; users of Khaini tobacco (tobacco mixed with slaked lime) from India, and other similar practices [28]-[31]. But comparative studies on individuals who consumed tobacco in different forms are scarce.

Also, only little attention has been given, until now, to the effect of different staining procedures on the results of micronuclei assays. An evaluation of the literature shows that a variety of different stains is used in micronuclei studies. Among the DNA-specific stains, the ones which are most widely used are Feulgen and acridine orange; in some experiments, 4¶,6-diamidino-2-phenylindole (DAPI) and propidium iodide were also used. About 30% of the studies on epithelial cells were conducted by using nonspecific stains (Giemsa, May- Grunwald's Giemsa, and less frequently, Orcein) [32]-[34]. Hence, the present study was done to fulfill the following study objectives:

1. To compare Papanicolaou's (Pap) and May Grunwald's Giemsa (MGG) staining techniques to detect micronuclei (MN) in exfoliated buccal mucosal cells in individuals with different tobacco habits.
2. To determine the severity of human buccal cell changes which are associated with smoking and smokeless (''chewing'') tobacco (SLT).

Materials and methods

Subjects

A total of 45 male subjects (15 smokers, 15 smokeless tobacco users and 15 non users/non smokers) were selected from among the outpatients who attended the Department of Oral and Maxillofacial Pathology, M M College of Dental Sciences and Research, Mullana, Ambala, Haryana, from January 2009 to October 2009. The smokers who smoked every day for at least five years and consumed >80 packs/year and smokeless tobacco chewers who chewed four or more packets daily for at least five years, were included in their respective groups. All were occasional drinkers who consumed alcohol once a week. The control group persons were not habituated to any form of tobacco consumption or pan chewing and were occasional drinkers who consumed alcohol once a week. The majority of them were living in rural areas.

Before collecting the samples, the written consent of each individual was taken. Each subject was asked about his lifestyle, food consumption, infectious diseases, X-ray exposure, medication, etc. Individuals who had had a recent viral infection or had been exposed to X-rays or those who had been under medication were excluded from the study. The lifestyle (except the habit of tobacco) and dietary habits of the controls were similar to those of the users.

Cytological preparations and examination

Before sampling, each individual rinsed his/her mouth thoroughly with tap water. The exfoliated cells were obtained by scraping the buccal mucosa with a moistened wooden spatula. The scraped cells were placed onto pre-cleaned slides. Two slides were made from each subject. One was air dried and stained with the MGG stain [Table/Fig 1], while the other was wet fixed and stained with PAP.

[Table/Fig 1]: Modified MGG staining method

Step No.	Step
1	Air dry the smear
2	Drain with May – Grunwald solution for 5 minutes
3	Drain with Giemsa solution for 15 minutes
4	Wash in running water

The slides were randomized and scored by a single observer. From each slide, ~ 1000 cells were examined under the 400X magnification and when MN cells were located, they were examined under the 1000X magnification. The criterion which was developed by Tolbert et al [Table/Fig 2] was used for counting the micronuclei.

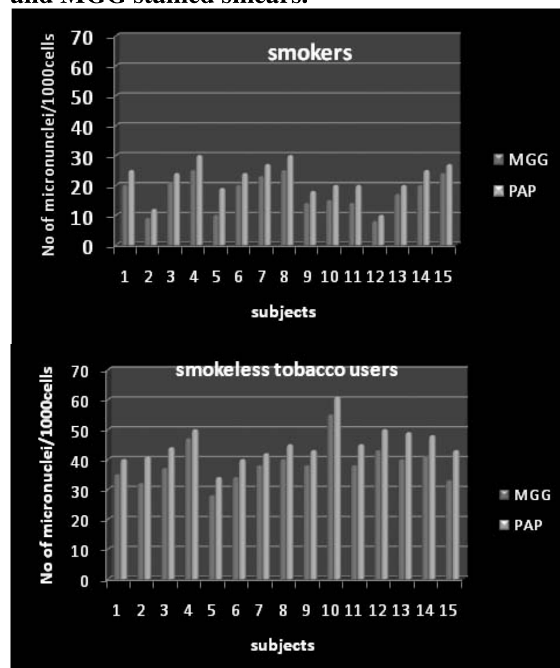
[Table/Fig 2]: Tolbert et al criteria

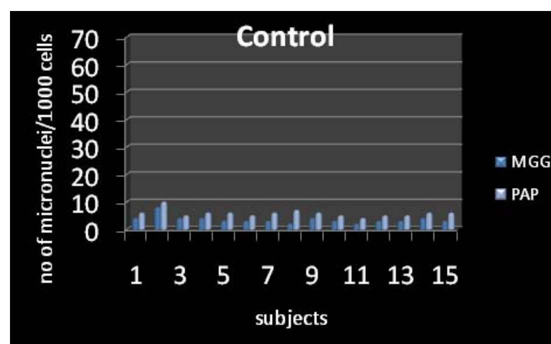
Parameters for cell inclusion in the cells to be scored:	The suggested criteria for identifying MN are:
1. intact cytoplasm and relatively flat cell position on the slide; 2. little or no overlap with adjacent cells; 3. little or no debris; and 4. nucleus normal and intact, nuclear perimeter smooth and distinct.	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. Feulgen positive, i.e. pink in bright field illumination; 4. staining intensity similar to that of the nucleus; 5. texture similar to that of nucleus; 6. same focal plane as nucleus; 7. absence of overlap with, or bridge to, the nucleus.

Results

The individual data is presented in [Table/Fig 3]. The frequency of cells with micronuclei was analysed by means of ANOVA, the one way analysis of variance.

[Table/Fig 3]: Frequency of distribution of micronuclei in buccal mucosal cells in both Pap and MGG stained smears.





The mean values which were obtained from the smokeless tobacco chewers, the smokers, the tobacco non users and the non smokers were compared in both the PAP and the MGG staining techniques [Table/Fig 4]. Bonferroni multiple comparisons were done amongst the different groups to determine the significance of the mean difference [Table/Fig 5].

[Table/Fig 4]: Mean number of micronucleated cells in different groups under different stains.

Group	Pap (mean +/- SD)	MGG (mean +/- SD)
Control	6.13+/- 2.29	3.53 +/- 1.407
Smokers	22.07 +/- 5.88	17.67 +/- 5.76
Smokeless tobacco chewers	45 +/- 6.18	38.6 +/- 6.51

[Table/Fig 5]: Multiple comparisons bonferroni

			Mean difference	Standard error	Significance
PAP	Control	Smokers	-15.933*	1.864	.001*
	Control	Smokeless tobacco users	-38.867*	1.864	.001*
	Smokeless tobacco users	Smokers	22.933*	1.864	.001*
			Mean difference	**Standard error**	**significance**
MGG	Control	Smokers	-14.133*	1.857	.001*
	Control	Smokeless tobacco users	-35.067*	1.857	.001*
	Smokeless tobacco users	Smokers	20.933*	1.857	.001*

* **The mean difference is significant at the .05 level**

The mean number of the micronuclei which was found among the control group by using the PAP stain was 6.13 +/- 2.29, whereas that which was obtained by using the MGG stain was 3.53 +/- 1.407. Similarly, the mean number of micronuclei among the smokers which was obtained by using the PAP stain was higher than that which was obtained by using the MGG stain i.e. 22.07 +/- 5.88 and 17.67 +/- 5.76, respectively. Again, among the smokeless tobacco chewers, the Pap stain smears showed more number of micronuclei

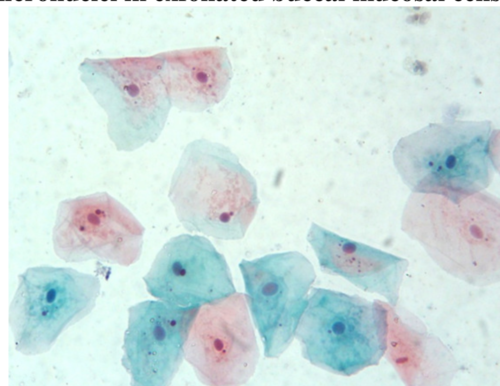
ie. 45 +/- 6.18 than the MGG stained smears, where the mean number of micronuclei was 38.6 +/- 6.51. As evident, the mean number of micronuclei cells was higher in the PAP stained smears in all the three groups than in the MGG stained smears. But the difference in the number of micronuclei in the MGG and PAP stained smears among the groups was not statistically significant. The mean number of micronuclei was significantly higher in smokeless tobacco users than in the smokers, non smokers and the non chewers in both the Pap and the MGG stained smears. Mutiple comparisons showed the mean difference to be highly statistically significant amongst the three groups in both the PAP and MGG stained smears [Table/Fig 5].

Discussion

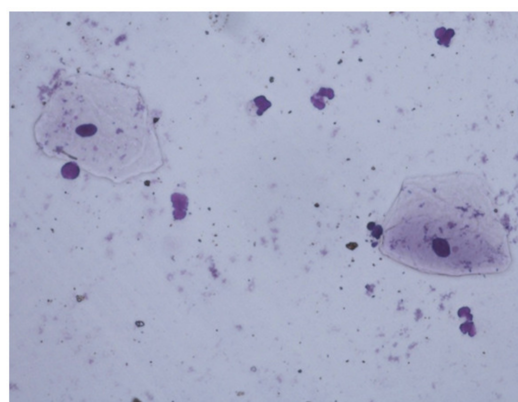
The usefulness of a micronucleus test to detect and quantitate the genotoxic action of carcinogens and mutagens has been well established in vitro as well as in vivo studies [35]. The sensitivity of the micronucleus test is comparable to that of scoring chromatid breaks and exchanges. A reasonable relationship between the carcinogenicity of chemicals and their capacity to induce micronuclei, as well as the ease of scoring, stimulated the application of the micronucleus test to exfoliated human cells. The MN assay can be used for exfoliated cells, which offers the advantage of conducting a genotoxicity test on material from an intact organism with its multitude of defense systems [35].

Our findings showed that the results of the micronuclei assay in exfoliated oral mucosal cells of tobacco users and non tobacco users depended strongly on the staining method. According to the results of the present study, for the routine micronuclei assay, PAP, which is the most commonly used cytological stain, was found to show better staining results as compared to the MGG, a Romanowsky's stain which is used widely in field studies. As for the PAP stain, the smear was wet fixed in alcohol which gave a clear background when compared to MGG, where the smear was air dried and resulted in a background which was full of cell debris and salivary proteins, thus masking the counting of the micronuclei [Table/Fig 6] and [Table/Fig 7].

[Table/Fig 6]: PAP stained smear showing micronuclei in exfoliated buccal mucosal cells.



[Table/Fig 7]: MGG stained smear showing micronuclei in exfoliated buccal mucosal cells



These findings were consistent with the findings of Sohair etal [36] who concluded that Pap stain was the preferred method in field studies for scoring and detecting MN in the cells of the buccal mucosa.

Hence, only Pap stained micronuclei values were used for multiple comparisons among the different groups.

As seen in the results section, the percentage of MN cells was significantly higher in smokeless tobacco users than in the non smokers/ non users and the mean difference between the two was statistically highly significant [Table/Fig 4] and [Table/Fig 5]. These findings are consistent with the recent studies of Sellapa et al [37] and Patel et al [38] where the MN count in smokeless tobacco users were higher than that in the control group. Carcinogenic and mutagenic compounds, including tobacco-specific nitrosamines, which are present in smokeless tobacco forms [39], are believed to be responsible for the induction of micronuclei.

These compounds are produced from nicotine by bacterial or enzymatic activity. The same formation occurs in the mouth under the influence of saliva [40].

In a study by Suhas et al [41] on buccal cell changes which are associated with smoking by using the micronucleus assay, there was found to be a significant correlation between the habit of smoking and the frequency of the micronucleated oral mucosal cells. The results of present study are in accordance with this study.

Ozkul etal [42] compared micronuclei formation in the buccal mucosal cells of habitual Maras powder (a form of smokeless tobacco) users with those of the smokers and found that the mean percentage of the micronuclated cells was 1.86+/- 0.26 in the users and 1.99+/-0.33 in the smokers. But there was no difference between themean percentages of the micronucleated cells in these two groups (p>0.05). In the present study, the mean percentage of the micronucleated cells was 4.5 +/- 0.61 in smokeless tobacco users while it was 2.20+/-0.5 in the smokers. The mean difference between the two groups was highly statistically significant.

The risk of cancer in smokeless tobacco users has been attributed to the presence of tobacco specific nitrosamines (TSNAs) [43]. There are four principal compounds: N-nitrosonornicotine (NNN), 4-methyl-N-nitrosamino-1-(3-pyridyl)-1-butanone (NNK), N-nitrosoanatabine (NAT) and N-nitrosoanabasine (NAB). Only two TSNAs, NNN and NNK, are considered to be potential carcinogens (IARC, 1985a). On the other hand, both NAT and NAB which are designated by IARC are not classifiable with regards to their carcinogenicity (IARC, 1985b). In India, SLT processing is performed by individual farmers and small companies with little control over fermentation and curing, which increases the production of TSNAs [43]. Also, SLT is not homogeneous in India, since the tobacco is often combined with betel leaf (Piper betle) and sliced areca nut (Areca catechu) and/or powdered slaked lime, which are additives that enhance the toxicity as well as the psychotropic effect of tobacco [44].

Also, studies have proven that the nicotine content of the Indian brands of smoking tobacco is slightly high as compared to that of the international brands [45]. The nicotine content in commercially available chewing tobacco products was found to be much lower than that in the smoking form of tobacco, but the average daily consumption has made it comparable to the smoking form [45].

According to the centre for disease control (CDC), chewing tobacco which was used 7-8 times a day could be equivalent to smoking 30-40 cigarettes per day. Other factors such as the use of slaked lime and continuous contact with the oral mucosa, led to more absorption of nicotine through smokeless tobacco use. Additionally, in contrast to the smokers who absorbed nicotine primarily through the pulmonary vasculature, chewing tobacco users were found to absorb nicotine through the buccal mucosa and the gastrointestinal tract mucosa.

Chewing tobacco is said to have increased the carcinogenic and genotoxic potential. The odds for oral cancer are estimated to be 7.3 in smokers, 1.3 in alcoholics and 11.4 in those who are habituated to chewing tobacco [46].

Conclusion

This study concludes that Pap is a better stain over MGG for the micronucleus assay screening of the buccal cells. The severity of the buccal changes which were associated with smokeless tobacco use was more than that in smokers, thus indicating the more genotoxic effect of smokeless tobacco. The limitation of this study was the sample size which could have been larger. This technique is primitive and further research by using fluorescent dyes and molecular markers is recommended.

References:

[1] Stich HF, San RH, Rosin MP. Adaptation of the DNA-repair and micronucleus tests to human cell suspensions and exfoliated cells. Ann. N.Y. Acad. Sci. 1983; 407:93–105.

[2] Fenech M, Holland N, Chang WP, Zeiger E, Bonassi S. The Human Micro-Nucleus Project—an international collaborative study on the use of the micronucleus technique for measuring DNA damage in humans. Mutat. Res. 1999; 428:271– 83.

[3] Fenech M, Crott JW. Micronuclei, nucleoplasmic bridges and nuclear buds induced in folic acid deficient human

lymphocytes-evidence for breakagefusion-bridge cycles in the cytokinesis-block micronucleus assay. Mutat. Res. 2002; 504:131– 6.
[4] Gonsebatt ME, Vega L, Salazar AM, Montero R, Guzman P, Blas J et al. Cytogenetic effects in human exposure to arsenic. Mutat. Res. 1997; 386:219– 28.
[5] Machado-Santelli GM, Cerqueira EM, Oliveira CT, Pereira CA. Biomonitoring of nurses handling antineoplastic drugs. Mutat. Res. 1994; 322:203– 8.
[6] Burgaz S, Karahalil B, Bayrak P, Taskin L, Yavuzaslan F, Bokesoy I et al. Urinary cyclophosphamide excretion and micronuclei frequencies in peripheral lymphocytes and in exfoliated buccal epithelial cells of nurses handling antineoplastics. Mutat. Res.1999; 439:97–104.
[7] Karahalil B, Karakaya AE, Burgaz S. The micronucleus assay in exfoliated buccal cells: application to occupational exposure to polycyclic aromatic hydrocarbons. Mutat. Res. 1999; 442:29–35.
[8] Celik A, Cavas T, Ergene-Gozukara S. Cytogenetic biomonitoring in petrol station attendants: micronucleus test in exfoliated buccal cells. Mutagenesis 2003; 18:417– 21.
[9] Aposhian HV. Enzymatic methylation of arsenic species and other new approaches to arsenic toxicity. Annu. Rev. Pharmacol. Toxicol. 1997; 37: 397– 419.
[10] Basu R, Woodruff TJ, Parker JD, Saulnier L, Schoendorf KC. Comparing exposure metrics in the relationship between PM2.5 and birth weight in California. J. Expo Anal. Environ. Epidemiol. 2004; 14:391– 6.
[11] Basu A, Mahata J, Roy AK, Sarkar JN, Poddar G, Nandy AK et al. Enhanced frequency of micronuclei in individuals exposed to arsenic through drinking water in West Bengal, India. Mutat. Res.2002; 516:29–40.
[12] Vuyyuri SB, Ishaq M, Kuppala D, Grover P, Ahuja YR. Evaluation of micronucleus frequencies and DNA damage in glass workers exposed to arsenic. Environ. Mol. Mutagen.2006; 47:562– 70.
[13] Tian D, Ma H, Feng Z, Xia Y, Le XC, Ni Z et al. Analyses of micronuclei in exfoliated epithelial cells from individuals chronically exposed to arsenic via drinking water in inner Mongolia, China. J. Toxicol. Environ. Health 2001; 64:473– 84.
[14] Gattas GJ, Soares-Vieira JA. Cytochrome P450-2E1 and glutathione S-transferase mu polymorphisms among Caucasians and mulattoes from Brazil. Occup. Med.2000; 50:508– 11.
[15] Ying CJ, Yan WS, Zhao MY, Ye XL, Xie H, Yin SY et al. Micronuclei in nasal mucosa, oral mucosa and lymphocytes in students exposed to formaldehyde vapor in anatomy class. Biomed. Environ. Sci.1997; 10:451– 5.
[16] Burgaz S, Erdem O, Cakmak G, Erdem N, Karakaya A, Karakaya AE. Cytogenetic analysis of buccal cells from shoe-workers and pathology and anatomy laboratory workers exposed to n-hexane, toluene, methyl ethyl ketone and formaldehyde. Biomarkers 2002; 7:151– 61.
[17] Benova D, Hadjidekova V, Hristova R, Nikolova T, Boulanova M, Georgieva I et al. Cytogenetic effects of hexavalent chromium in Bulgarian chromium platers, Mutat. Res. 2002; 514:29–38.
[18] Torres-Bugarin O, Ventura-Aguilar A, Zamora-Perez A, Gomez-Meda BC, Ramos-Ibarra ML, Morga-Villela G, et al. Evaluation of cisplatin + 5-FU, carboplatin + 5-FU, and ifosfamide + epirubicine regimens using the micronuclei test and nuclear abnormalities in the buccal mucosa, Mutat. Res. 2004; 565:91–101.
[19] Martino-Roth MG, Viegas J, Roth DM. Occupational genotoxicity risk evaluation through the comet assay and the micronucleus test. Genet. Mol. Res. 2003; 2:410– 7.
[20] Diaz S, Fonseca G, Fernandez I. Analysis of lymphocyte and oral mucosa cell micronuclei in Cuban paint industry workers. Hereditas 1990; 113:77–80.
[21] Goud KI, Hasan Q, Balakrishna N, Rao KP, Ahuja YR. Genotoxicity evaluation of individuals working with photocopying machines. Mutat. Res. 2004; 563:151– 8.
[22] Sailaja N, Chandrasekhar M, Rekhadevi PV, Mahboob M, Rahman MF, Vuyyuri SB, et al. Genotoxic evaluation of workers employed in pesticide production. Mutat. Res.2006; 609:74–80.
[23] Gomez-Arroyo S, Diaz-Sanchez Y, Meneses-Perez MA, Villalobos-Pietrini R, De Leon-Rodriguez J. Cytogenetic biomonitoring in a Mexican floriculture worker group exposed to pesticides. Mutat. Res.2000; 466:117– 24.
[24] Pinto D, Ceballos JM, Garcia G, Guzman P, Del Razo LM, Vera E, et al. Increased cytogenetic damage in outdoor painters. Mutat. Res. 2000; 467:105– 11.
[25] Chen C, Arjomandi M, Qin H, Balmes J, Tager I, Holland N. Cytogenetic damage in buccal epithelia and peripheral lymphocytes of young healthy individuals exposed to ozone. Mutagenesis 2006; 21:131– 7.
[26] Gonsebatt ME, del Valle M, Fourtoul T, Pinto D, Ceballos JM, Garcia G. Micronucleus (MN) frequency in nasal respiratory epithelium from young adults living in urban areas with different levels of air pollution. Mut. Res.1997;379:398.
[27] Huen K, Gunn L, Duramad P, Jeng M, Scalf R, Holland N. Application of a geographic information system to explore associations between air pollution and micronucleus frequencies in African American children and adults. Environ. Mol. Mutagen.2006; 47:236–46.
[28] Bonassi S, Neri M, Lando C, Ceppi M, Lin YP, Chang WP, et al. Effect of smoking habit on the frequency of micronuclei in human lymphocytes: results from the Human MicroNucleus project. Mutat. Res.2003; 543:155– 66.

[29] Tolbert PE, Shy CM, Allen JW. Micronuclei and other nuclear anomalies in buccal smears: a field test in snuff users. Am. J. Epidemiol.1991; 134:840– 50.
[30] Das RK, Dash BC. Genotoxicity of 'gudakhu', a tobacco preparation. II. In habitual users. Food Chem. Toxicol.1992; 30:1045– 9.
[31] Trivedi AH, Dave BJ, Adhvaryu SG. Monitoring of smokeless tobacco consumers using cytogenetic endpoints. Anticancer Res. 1993; 13:2245– 9.
[32] Nair U, Obe G, Nair J, Maru GB, Bhide SV, Pieper R et al. Evaluation of frequency of micronucleated oral mucosa cells as a marker for genotoxic damage in chewers of betel quid with or without tobacco Mutat. Res.1991; 261:163- 8.
[33] Casartelli G, Monteghirfo S, De Ferrari M, et al. Staining of micronuclei in squamous epithelial cells of human oral mucosa. Anal Quant Cytol Histol 1997; 19:475- 81.
[34] Nersesyan A, Kundi M, Atefie K, Schulte-Hermann R, Knasmuller S. Effect of Staining Procedures on the Results of Micronucleus Assays with Exfoliated Oral Mucosa Cells. Cancer Epidemiol Biomarkers Prev 2006; 15(10).
[35] Hayashi M, Sofini T, Ishidate Jr. M. An application of acridine orange fluorescent staining to the micronucleus test. Mutat. Res.1983; 120:241– 7.
[36] Ayyad SBA, Israel E, El-Setouhy M, Nasr GR, Mohamed MK, Loffredo CA. Evaluation of Papanicolaou stain for studying micronuclei in buccal cells under field conditions. Acta cytologica.2005; 50(4):398-402.
[37] Sellappa S, Balakrishnan M, Raman S, Palanisamy S. Induction of micronuclei in buccal mucosa on chewing a mixture of betel leaf, areca nut and tobacco. Journal of Oral Science 2009; 51(2): 289- 92.
[38] Patel BP, Trivedi PJ, Brahmbhatt MM, Shukla SN, Shah PM, Bakshi SR. Micronuclei and chromosomal aberrations in healthy tobacco chewers and controls: A study from Gujarat, India. Arch Oncol 2009; 17(1-2):7-10.
[39] Hecht SS and Hoffmann D. Tobacco-specific nitrosamines. An important group of carcinogens in tobacco and tobacco smoke. Carcinogenesis 1988; 9: 875- 84.
[40] Winn DM, Blot WJ, Shy CM, Pickle XW, Toledo A, Fraumeni JF. Snuff dipping and oral cancer among women in the Southern United States. N. EngL J. Med.1981; 304:745- 8.
[41] Suhas S, Ganapathy KS, Gayatridevi, Ramesh C. Application of the micronucleus test to exfoliated epithelial cells from the oral cavity of beedi smokers, a high-risk group for oral cancer. Mutation Research 2004; 561:15–21.
[42] Ozkul Y, Donmez H, Erenmemisoglu A, Demirtas H, Imamoglu N. Induction of micronuclei by smokeless tobacco on buccal mucosa cells of habitual users. Mugenesis 1997; 12(4):285- 7.
[43] Rodu B, Jansson C. Smokeless tobacco and oral cancer: A Review of the risks and determinants. Crit Rev Oral Biol Med 2004; 15(5):252- 63.
[44] Main JH, Lecavalier DR. Smokeless tobacco and oral disease: A review. J Can Dent Association 1988; 54:586- 91.
[45] Sujatha S Reddy, Shaik Hyder Ali KH. Estimation of nicotine content in popular Indian Brands of smoking and chewing tobacco products. Indian J Dent Res 2008; 19(2):88-91.
[46] Prabhakaran PS, Mani N. Epidemiology of oral cancer. Oral oncology CME annual Kidwai Memorial Institute of Oncology: Bangalore; 2002.p. 1- 10.