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Genotoxicity of tobacco and alcohol on human oral mucosal cells

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ABSTRACT

Chewing and smoking of tobacco as well as drinking of alcohol have been reported as the risk factors for oral cancer. The high incidence of oral cancer in India and gradual increase of oral cancer patients at the Out Patient Department of Acharya Harihar Regional Cancer Center (AHRCC) Cuttack, Odisha (the only Government Hospital of the state dedicated for the treatment of the cancer patients) prompted us to undertake this study. In this hospital-based case-control study, the genotoxic effect of tobacco and alcohol was evaluated through micronucleus test. The percentage of micronucleated cells were recorded to be the highest in chewer-smoker-alcoholics group (2.86 in males and 3.35 in females) and the least in smokers' group (0.68 in males and 0.65 in females). The values were found to be statistically significant at 1% level of confidence (Z-test). But, the occurrence of micronucleated cells in higher percentage in non-addicted cancerous group (1.23 in males and 1.20 in females) than the single addicted groups (such as chewers, smokers and alcoholics) indicate the causes of genetic susceptibility followed by oral unhygienic condition and modern life style in food pattern among the people of Odisha. Therefore, it needs special attention for which further investigation is suggested.

Key words: Genotoxicity, oral cancer, buccal mucosa, exfoliated cells, cytosmears, addiction, micronucleus.

INTRODUCTION

Oral cancer is one of the ten most common cancers as stated by World Health Organization (WHO) and each year 5,75,000 new cases and 3,20,000 deaths occur world-wide [1]. In India, oral cancer is a major health problem which accounts for 30-40% of all cancers diagnosed and is the sixth common cause of death in males and seventh in females [2]. In Orissa, tobacco related oral cancer is found to be very common which may be due to the chewing habit of different forms of tobacco (oral snuff, khaini, betel, betel-quid, gutkha and paan masala) and smoking of bidi (a type of native cigarette prepared from dried tobacco leaves powder, rolled in a piece of tembhurni leaves), cigarettes etc. Registration of more number of late-stage oral cancer patients for first time treatment in the Out Patient Department (OPD) of Acharya Harihar Regional Cancer Center (AHRCC), Cuttack, and Odisha has prompted us to undertake this study. The present investigation aims at the study of genotoxicity of tobacco and alcohol on the buccal mucosa of oral cancer patients and to assess the relevance of micronucleus test in the early detection of oral cancers.

MATERIALS AND METHODS

2.1 Collection of samples

Exfoliated cytosmears were collected by scraping from the clinically diagnosed 136 patients suffering from precancerous lesions and oral squamous cell carcinoma (OSCC) at the Out Patient Department (OPD) of Acharya Harihar Regional Cancer Center (AHRCC), Cuttack, Odisha, during May 2007-May 2009. Smearing was done on the pre-cleaned-coded microslides and the slides were fixed in aceto-alcohol (1:3) fixative, immediately. Two slides were smeared and prepared from each affected sites of the patient. Prior to the collection of samples, case-history of the patients related to their age, sex, food, habits, oral hygiene and occupation were asked and recorded for detail analysis. A parallel set of 136 samples were also collected from the non-addicted and non-cancerous individuals from different regions of Odisha which are called as control group.

2.2 Staining protocol and scoring of micronuclei

The wet fixed smears were stained by adopting Papanicolaou's staining protocol and counter-stained with Giemsa's solution. One thousand cells were screened and the micronucleated cells were scored from each stained sample following standard criteria [3,4,5]. Although more than one micronuclei were observed in oral squamous cells (Figs.1-4), the scored elements were MNCs and not the number of micronuclei [6]. Photomicrographs were taken out as the supporting evidence.

2.3 Statistical analysis

Findings were statistically analyzed, interpreted and correlated with the age group, sex and degree of pathogenicity. Test of proportion (Z-test) was followed and the critical ratio (Z-value) was calculated for the test of significance. The following formula was used for the calculation of Z-value [7].

$$Z = \frac{Ip_1 - p_2 I}{\sqrt{\frac{p_1 q_1}{n_1} + \frac{p_2 q_2}{n_2}}}$$

Where, p_1 = Proportion of the MNCs in control group (sample-I), p_2 = Proportion of the MNCs in affected group (sample-II), q_1= 1-p_1 (in case of percentage, 100-p_1), q_2=1-p_2 (in case of percentage, 100-q_2), n_1=size of the sample-I, n_2= size of the sample-II,

and $\sqrt{\frac{p_1 q_1}{n_1} + \frac{p_2 q_2}{n_2}}$ = Standard error of difference between the two sample proportions.

The observed Z-values for normal distribution at 5% and 1% level of confidence are 1.96 and 2.576 respectively.

RESULTS

The general characteristics of the oral cancer cases such as age group, food habit, addiction, occupation and oral hygiene were taken into account (Table.1). Out of 136 patients, 82 (60.3%) were males and 54 (39.7%) were females. More number of oral cancer patients were observed in 50-69 years of age group than the other two groups. The highest percentages of the OSCC patients were recorded to be 41.5 and 66.7 in males and females respectively, whereas, the lowest percentages were found to be 18.3% in males and 12.9% in females in the age group of 70-89 years.

Basing on the food habits of the patients, 92.7% were non-vegetarian who prefer to take meat, fish and eggs while 7.3% were vegetarian. In non-vegetarian group, the percentage of males was more (96.3%) than the females (87.1%). On the contrary, the percentage of females is more (12.9%) than the males (3.7%) under vegetarian category.

It is reported that out of 136 oral cancer patients, 126 (92.6%) were addicted to different forms of tobacco and alcohol for more than 15 years, while 10 (7.4%) were non-addicted completely. The addicted individuals were categorized into five different groups, such as chewers, smokers, alcoholics, chewer-smokers and chewer-smoker-alcoholics. The chewers' group having 43 patients was recorded to be the highest (31.61%) and the smokers' group having 11 patients was found to be the lowest. All the patients were reported to brush their teeth at least once in a day, but in different ways.

Table 1 Sex-wise general characteristics of the oral cancer cases

		Number of cases	**%**	**Number of cases**	**%**	**Number of cases**	**%**
1	**Age group**						
	30-49	33	40.2	11	20.4	44	32.4
	50-69	34	41.5	36	66.7	70	41.4
	70-89	15	18.3	07	12.9	22	16.2
2	**Food habit**						
	Vegetarian	03	3.7	07	12.9	10	7.4
	Non-vegetarian	79	96.3	47	87.1	126	92.6
3	**Addiction**						
	Chewers	23	27.8	20	37.02	43	31.61
	Smokers	09	10.9	02	3.7	11	8.08
	Alcoholics	12	14.7	18	33.3	30	22.05
	Chewer-smokers	24	29.5	04	7.4	28	20.58
	Chewer-smoker- alcoholics	08	9.8	06	11.2	14	10.29
	Non-addicted	06	7.3	04	7.4	10	7.35
4	**Occupation**						
	Labourer	17	20.7	22	40.7	3.9	28.7
	Farmer	08	9.7	Nil	Nil	08	5.8
	Teacher	15	18.3	03	5.6	18	13.2
	Others(heterogenous)	42	51.3	29	53.7	71	52.3
5	**Oral hygiene: Tooth brushing**						
	Plant stick only	36	43.9	26	48.1	62	45.5
	Plant stick+ toothpaste/powder	15	18.2	09	16.6	24	17.6
	Tooth brush with paste/powder	31	37.9	19	34.3	50	36.9

Source: Primary data.

The genotoxicity of tobacco and alcohol on buccal mucosal cells of human being has been evaluated through micronucleus test (MNT). Because, MNT is one of the most widely applied short term test used in genetic toxicology and has become one of the most important tests implemented by the regulatory authorities of different countries to evaluate mutagenicity of, and sensitivity to, xenobiotics [8, 9]. Since, the formation of micronuclei in the eukaryote cells is an end point of chromosomal damage or segregation errors [10], the presence of micronuclei reflects a genotoxic exposure. The micronucleated cells were observed to have either one or more than one micronuclei (Figs.1-4). In this present study, the scored elements were micronucleated cells and not the number of micronuclei [3, 4, 6].

The frequencies of micronucleated cells (MNCs) in control and addicted cancerous and non-addicted cancerous groups exhibit an increasing order from lower to higher age groups (Table 2). In control group, all of 82 males and 54 females were non-addicted and non-cancerous individuals. In the age group of 30-49, 50-69 and 70-89 years, the number and percentage of MNCs scored from 33, 34 and 15 males were 07, 09 and 06 and 0.021, 0.026 and 0.068 accordingly. The number and frequencies of MNCs from 11, 36 and 07 females were scored to be 02, 07 and 02 and 0.018, 0.019 and 0.028 in 30-49, 50-69 and 70-89 years of age groups, respectively (Figs.5-8). The mean percentage was calculated to be 0.026 in males and 0.020 females respectively.

The MNCs in chewers' group (Table 2) were scored to be 141, 99 and 109 from 11,07 and 05 samples of male individuals having the percentage 1.28, 1.41 and 2.18 in 30-49, 50-69, 70-89 years of age group respectively. The mean percentage of MNCs in male was calculated to be 1.51. The scoring of MNCs in females were recorded to be 63, 137 and 82 from 06, 11 and 03 females with the percentage of 1.05, 1.25 and 2.73 in the age group of 30-49, 50-69 and 70-89 years, respectively having the mean percentage 1.41. In comparison with the control group, the critical ratios were found to be 18.409 in males and 16.628 in females and are thus highly significant ($p < 0.01$).

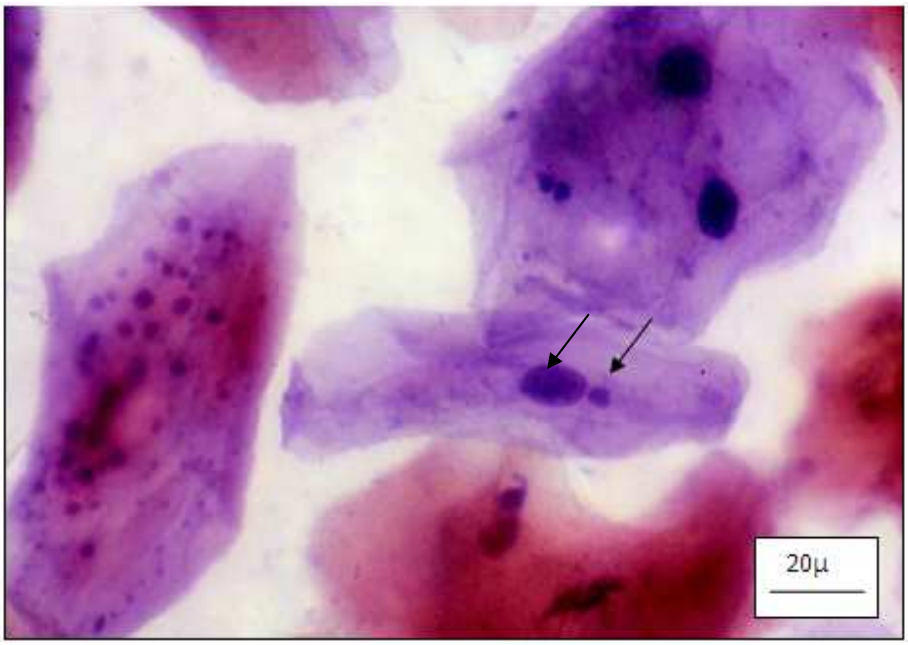

Fig.1 Monomicronucleated oral squamous cell (Giemsa's stain x400)

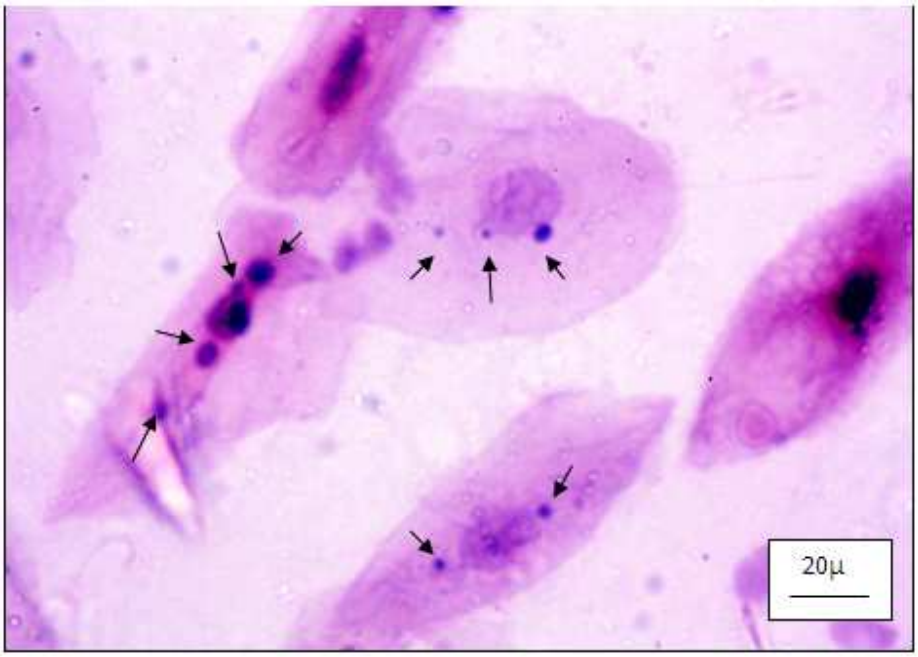

Fig.2 Bi-, tri- and tetramicronucleated condition of oral squamous cells (Giemsa's stain, x400)

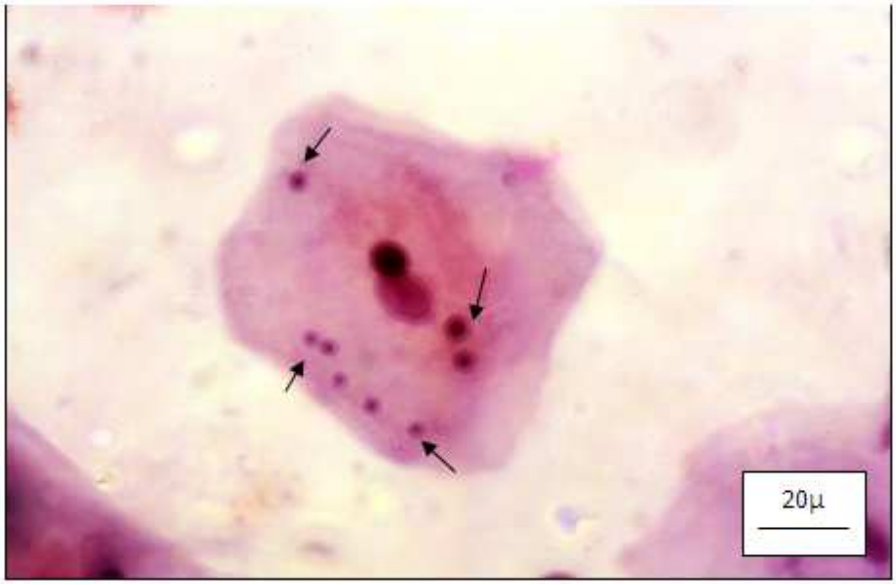

Fig.3 Anisonucleated octamicronucleated condition of an oral squamous cell (Papanicolaou's stain, x 400)

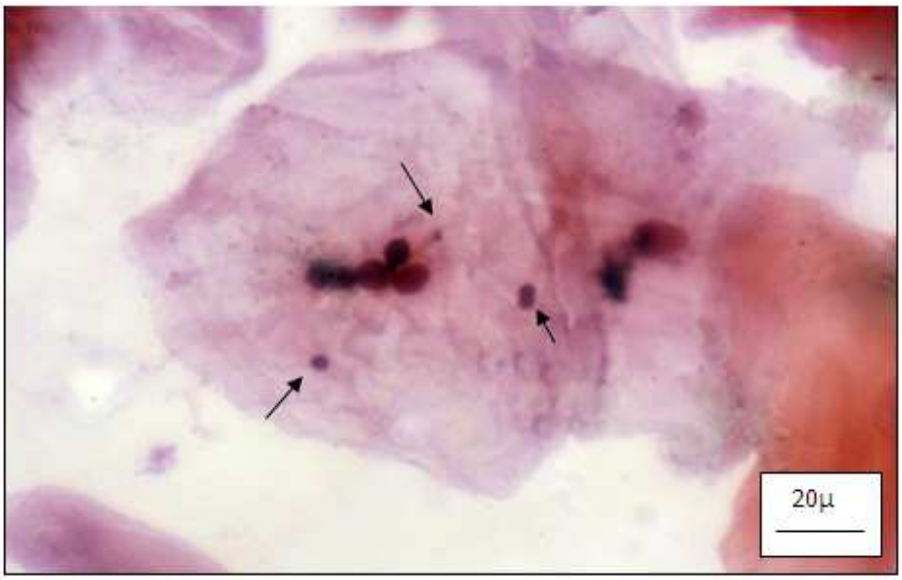

Fig.4 Pentanucleated trimicronucleated condition of an oral

squamous cell (Papanicolaou's stain, x 400).

The number and frequencies of micronucleated cells in smokers' group were observed to be 12 and 0.60 in 02 males in the age group 30-49 years. No female cases were reported to register in this age group. In 50-69 years, 32 MNCs from 05 males and 13 MNCs from 02 females having percentage 0.64 and 0.65 were scored respectively. In 70-89 years, no female cases were reported and hence 28 MNCs were enumerated from 02 males having the percentage 0.90. Thus, the mean percentage of MNCs in males and females were calculated to be 0.680 and 0.65 respectively. In smokers' group, the critical values were calculated to be 7.53 and 3.504 in males and females respectively (Figs.5-8). Since, these values are greater than the normal observed value (Z=2.576), it indicates that smoking has a genotoxic effect on buccal mucosa for the development of oral carcinoma.

In alcoholic group, 24, 40 and 28 numbers of MNCs were calculated from 04, 05 and 03 males having the percentage of 0.60, 0.80 and 0.93 in the age group of 30-49, 50-69 and 70-89 years respectively. The mean percentage of MNCs in males was 0.76. The number and frequencies of MNCs in females were scored to be 22, 87, 19 and0.55, 0.725, 0.95 from 04, 12 and 02 females in 30-49, 50-69 and 70-89 years of age groups. The mean percentage of the MNC was calculated to be 0.71 in females. The Z-values, in alcoholic groups are, thus calculated to be 9.235 in males and 10.937 in females (Figs.5-8), which implies that alcohol has a synergistic effect on oral mucosa in the process of micronuclei formation followed by oral carcinogenesis.

Referring to the chewer-smokers group, the MNCs were scored to be 142 from 09 males with a frequency of 1.571 per cent in 30-49 years of age group. In this age group, no MNCs were scored from the females due to unreported female oral cancer patients. In 50-69 years of age group, 215 and 63 MNCs were scored from 12 males and 03 females. The calculated frequencies were 1.79 in males and 2.10 in females. The number and frequencies of MNCs were reported to be 71 and 2.36 per cent in 03 males and 23 and 2.30 per cent in one female of 70-89 year age group. The average frequencies were calculated to be 1.78 in males and 2.15 in females. The critical ratios in chewer-smokers group were recorded to be 20.506 in males and 9.284 in females (Figs.5-8). It signifies that both chewing and smoking of tobacco have a greater impact on oral squamous cells than those of chewers or smokers.

The number and frequencies of MNCs in chewer-smoker-alcoholics group were observed to be very high. It was estimated that, 86 MNCs from 04 males having 2.15 per cent frequency was calculated in the 30-49 years of age group. Female cases were not recorded in this age group because of unreported situation. In 50-69 years, the number and frequencies of MNCs observed from 03 males were 106 (3.53 per cent) and from 06 females, 213(3.55 per cent) respectively. No female but only 01 male was recorded in 70-89 years of chewer-smoker-alcoholics group. The number and percentage of MNCs were found to be 37 and 3.70 in this age group respectively. Hence, the average percentage of MNCs in males was 2.802 and in females, it was 3.55. The Z-values in this group was calculated to be 15.20 in males and 14.772 in females and were found to be significantly higher than the normal value (Z=2.576) at 1% level of significance (Figs.5-8).

In non-addicted cancerous group, the numbers of MNCs were reported to be 17, 34 and 23 in 03, 02 and 01 male and having the percentage 0.56, 1.70 and 2.30 in 30-49, 50-69 and 70-89 year of age groups respectively. The mean percentage of MNCs in males was calculated to be 1.23. In females, 07, 26 and 15 MNCs were reported from 01, 02 and 01 females with the frequencies of 0.70, 1.30 and 1.50 in 30-49, 50-69 and 70-89 years of age group. In females, the mean percentage of the MNCs was calculated to be 1.20. The Z-values, in non-addicted cancerous group were calculated to be 8.454 in males and 6.849 in females and were found to be significant ($p<0.01$). Though, very few non-addicted individuals (06 males and 04 females) suffer from oral carcinoma, the existence of significant number of micronuclei in their exfoliated samples were, probably, due to genetic susceptibility, oral unhygienic, modern life style in food habit or even multifactorial effect.

Table 2 Frequencies of micronucleated cells (MNCs) in control, addicted and non-addicted cancerous groups

No	Group	Age group in years	Number of Samples screened		Number of cells MNCs scored		Percentage of MNCs		Mean percentage of MNCs		Critical ratio (Z-value)*	
			Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
1	Control	30-49	33	11	07	02	0.021	0.018	0.026	0.020	-	-
		50-69	34	36	09	07	0.026	0.019				
		70-89	15	07	06	02	0.068	0.028				
Total		30-89	82	54	22	11	0.026	0.020	0.026	0.020	-	-
2.	Chewers	30-49	11	06	141	63	1.28	1.05	1.51	1.41	18.409*	16.628*
		50-69	07	11	99	137	1.41	1.25				
		70-89	05	03	109	82	2.18	2.73				
3	Smokers	30-49	02	Nil	12	Nil	0.60	Nil	0.68	0.65	7.53*	3.504*
		50-69	05	02	32	13	0.64	0.65				
		70-89	02	Nil	18	Nil	0.90	Nil				
4.	Alcoholics	30-49	04	04	24	22	0.60	0.55	0.76	0.71	9.235*	10.973*
		50-69	05	12	40	87	0.80	0.725				
		70-89	03	02	28	19	0.93	0.95				
5.	Chewer- Smokers	30-49	09	Nil	142	Nil	1.57	Nil	1.78	2.15	20.506*	9.284*
		50-69	12	03	215	63	1.79	2.10				
		70-89	03	01	71	23	2.36	2.30				
6.	Chewer- smoker-alcoholics	30-49	04	Nil	86	Nil	2.15	Nil	2.86	3.55	15.20*	14.772*
		50-69	03	06	106	213	3.53	3.55				
		70-89	01	Nil	37	Nil	3.70	Nil				
7.	Non-addicted cancerous	30-49	03	01	17	07	0.56	0.67	1.23	1.20	8.454*	6.849*
		50-69	02	02	34	26	1.70	1.30				
		70-89	01	01	23	15	2.30	1.50				
Total		30-89	82	54	1234	770	1.504	1.425	1.504	1.425	34.472*	27.353*

*Source: Primary data. * Significant at 1% level ($p<0.01$) of confidence, where tabulated figure of $Z=2.576$.*

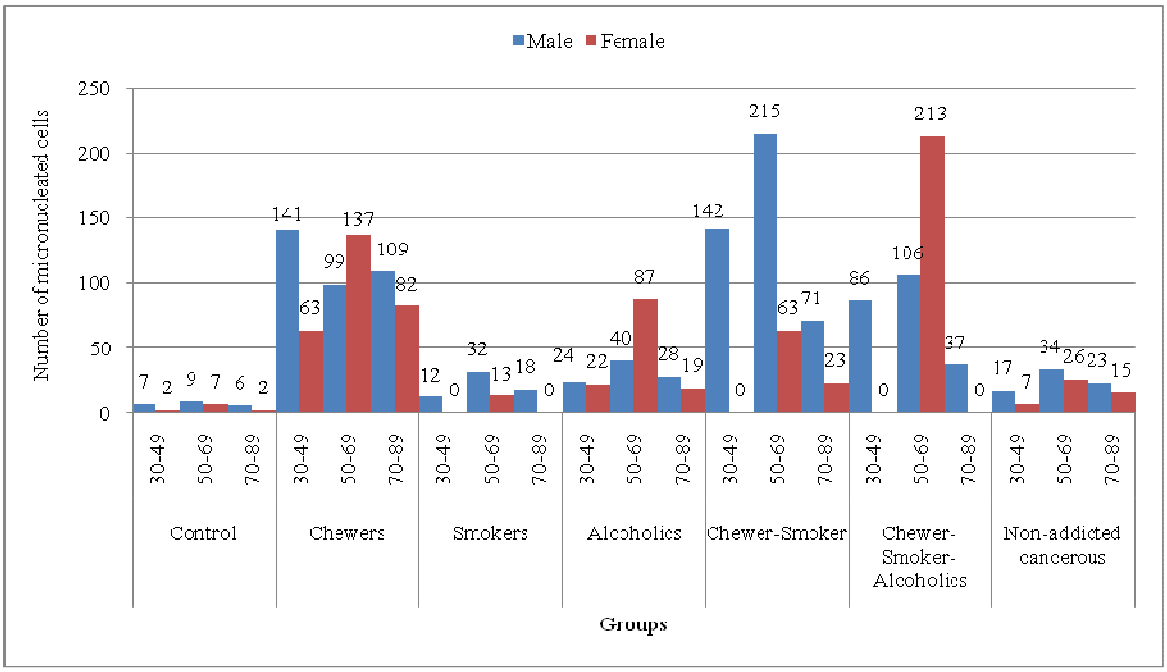

Fig.5 Age group and addiction-wise enumeration of micronucleated cells

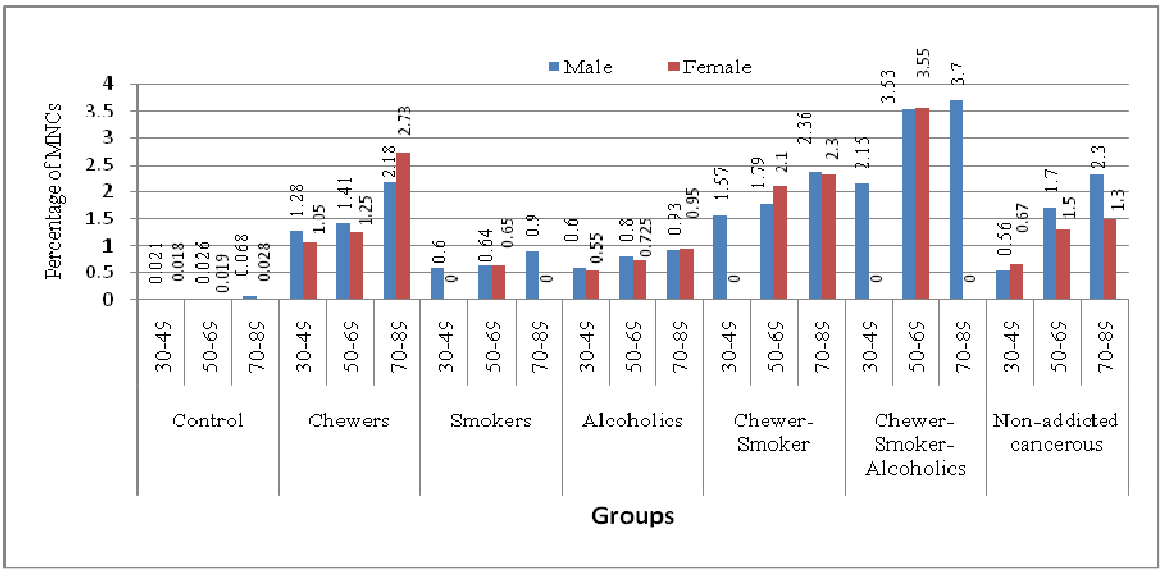

Fig.6 Percentage of micronucleated cells in different age groups of normal,addicted and non-addicted cancerous groups

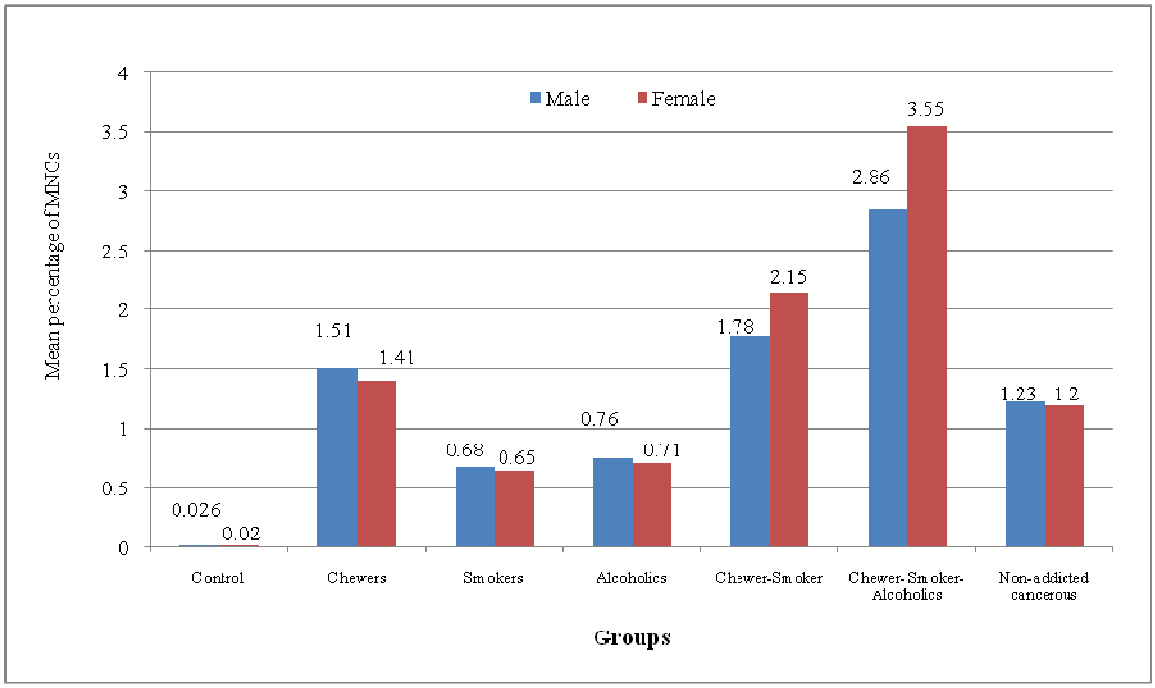

Fig.7 Age group and sex-wise mean percentage of micronucleated cells in control, addicted and non-addicted cancerous groups

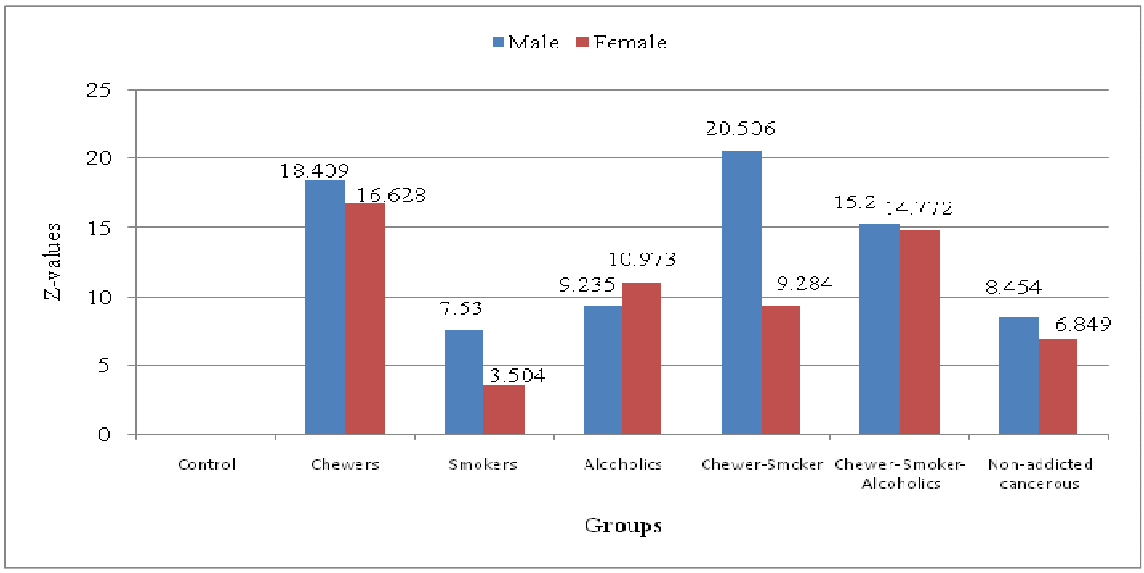

Fig.8 Critical ratios(Z-values) in addicted and non-addicted cancerous groups

Looking into the above analysis, it was observed that the number and frequency of MNCs were in increasing trends along with the increase of age, in both control and addicted groups. It has also been observed that the mean frequencies of MNCs were recorded to be the lowest (0.68 in males and 0.65 in females) in smoker group and the highest (2.86 in males and 3.55 in females) in chewer-smoker-alcoholic group. But, the frequency of MNCs in non-addicted cancerous group (1.23 per cent in males and 1.20 per cent in females) was found to be more than those of smokers (0.68 per cent in males and 0.65 per cent in females) and alcoholics group (0.76 per cent in males and 0.71 per cent in females) which needs further investigation. The present study corroborates the earlier findings of [11, 12, 13].

Micronucleated cells are, no doubt, a type of pleomorphic diagnostic cell in oral squamous cell carcinoma (OSCC) having its own significance. Formation of micronuclei in the epidermal squamous cells is a clear indication of genetic alteration in these cells. In the present study, it has been found that tobacco and alcohol are not the only factors of micronucleation but some other causes responsible for this may be genetic, environmental, biological and physical factors. During this investigation, it was established that longer the duration of abuse of tobacco and alcohol, greater was the number of MNCs. It has also been proved through the test of significance that chewing and smoking of tobacco and drinking of alcohol enhance the rate of formation of micronuclei along with other cytological atypias followed by oral squamous cell carcinoma (OSCC).

DISCUSSION

Micronuclei are either acentric chromosome fragments or whole chromosome lagging in the normal karyokinesis [4, 6, 14]. These laggards or fragments of chromosomes are excluded from the post-mitotic daughter cells and form nuclei. Thus, these micronucleated cells (MNCs) are represented either chromosomal breakage (clastogenicity) or a failure in the spindle fibre mechanism (tubragenicity) during cell division.

The induction of micronucleus in oral mucosal cells seems to be due to the presence of genotoxic agents in the tobacco and alcohol. According to Livingstone *et al.* [15] , saliva soluble compounds present in tobacco could diffuse into the basal cell layer and disturb the reproductive mechanism of the underlying proliferating cell population, thereby bringing about genotoxicity and micronuclei formation. Nagler and Dayan [16] have also reported that the interaction between redox active metals in saliva and the low reactive free radicals during chewing and smoking of tobacco enhance the potency of genotoxicity. The result may be that saliva loses its antioxidant property and instead, it becomes a potent pro-oxidant milieu. This leads to oral mucosal cells to be an aberrant one and promotes for oral carcinogenesis.

It has also been reported that chewing of betel-quid produces reactive oxygen species (ROS) that have multiple detrimental effects upon the oral mucosa. The production and release of ROS occurs under alkaline condition, during the auto-oxidation of areca-nut polyphenols in the saliva of betel-quid-chewers [17] . Prokopczyk *et al.* [18] have opined that , areca-nut- specific-nitrosamines along with tobacco-specific-nitrosamines (TSNA) are found to be mutagenic, genotoxic, and carcinogenic and capable of inducing tumors in the oral cavity.

Alcohol, acting both independently as well as synergistically with smoking has been implicated in oral carcinogenesis [19]. More importantly, alcohol may act as a solvent and enhance the penetration of carcinogens into target tissues. In fact, alcohol (ethanol) is metabolized by an enzyme alcohol dehydrogenase and to some extent, by cytochrome p^{450} to acetaldehyde which is found to be carcinogenic [20, 21]. The growing evidences associate increased alcohol consumption with risk of developing OSCC [22, 23]. The combined effects of tobacco use and alcohol consumption are found to be multiplicative. Compared with persons who neither drink nor smoke, the risk of developing OSCC is increased 80 fold in persons with the highest level of smoking and alcohol consumption [24].

Earlier reports suggest that the micronucleus test in human exfoliated oral mucosal cells provides evidence of exposure to carcinogens and measure of degree to carcinogen exposure in the tissue from which cancer develops. Increase in micronucleus frequency in buccal mucosal cells of tobacco and alcohol-users indicates a high risk group of oral cancer [11, 12, 14, 25, 26]. Gandhi and Kaur [13] have reported that the mean frequency of MNCs among paan masala chewers was 0.303±0.058 and was found to be significant when compared with the control group (mean frequency was nil), but non-significant among the different age groups of paan masala chewers. Some authors have described sex as an important variable in the micronucleus test [27, 28, 29], with males generally being more sensitive than females to the induction of micronuclei [30, 31]. But, other studies have shown no sex related differences in micronucleus test results [32, 33, 34, 35].

In the present study, the mean percentages of the MNCs were found to be significantly the highest (2.86 in males and 3.35 in females) in chewer-smoker-alcoholics group and the lowest (0.68 in males and 0.65 in females) in smokers group. It is also observed that, the mean percentage of MNCs was calculated to be more in chewers group (1.15 in males and 1.41 in females) than the alcoholics group (0.76 in males and 0.71 in females). But, the recorded percentage of MNCs in chewer-smokers group (1.78 in males and 2.15 in females) was found to be higher than the

independent groups of chewers and smokers. The present finding indicates that the combined effect of tobacco and alcohol is proved to be highly genotoxic to the buccal mucosal cells in both sexes. No doubt, the genotoxic effect of smoking is observed to be the lowest, but it enhances the formation of micronuclei in the buccal mucosal cells of human being along with the chewing of tobacco and drinking of alcohol. The percentage of MNCs in non-addicted cancerous group was found to be more (1.23 in males and 1.20 in females) than the chewers, smokers and alcoholics groups, but less than the mixed groups, which may be due to oral unhygiene, modern life style (mostly consuming fast food) and genetic susceptibility, which needs special attention for further research.

CONCLUSION

The present investigation was a hospital-based case-control study. The findings indicate that the combined effect of tobacco and alcohol has shown more genotoxicity than the single use on the buccal mucosal cells. The formation of more number of micronucleated cells in chewer-smoker-alcoholics group followed by in chewer-smokers than other addicted (chewers, smokers and alcoholics) groups has proved the genotoxic effect of tobacco and alcohol on oral mucosal cells. The results also support the facts of earlier findings that longer the addiction, more the number of micronuclei formation in oral mucosal cells. Furthermore, higher percentage of micronucleated cells in non-addicted oral cancer patients has proved the micronucleated cells to be an onco-indicator and micronucleus test to be the simplest tool for the test of genotoxicity as well as for the early detection of cancer of the oral cavity.

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