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Prognostic Significance of Micronuclei Assay in Predicting the Relative Risk of Cancer in Buccal Mucosa in High Risk Population

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Abstract

Background: Micronucleus in buccal mucosa exfoliative cells is essential to have reliable and relevant minimally invasive biomarkers to improve the implementation of biomonitoring, diagnostics, and treatment of disease caused by, or associated with genetic damage. *Methods:* The present retrospective study was carried in 100 persons in which 25 persons were alcohol consumers, 25 persons were tobacco smokers, 25 persons were both alcohol consumers and tobacco smokers and 25 persons were control group, in the department of pathology, Narayana Medical College & Hospital, Nellore, Andhra Pradesh, India, during the period of January 2016 to December 2016. Cytological smears was fixed in ethyl alcohol and then stained with Hematoxylin & Eosin staining technique and assessed for micronuclei count. *Result:* The micronucleus count in "A" group was a mean count of 4.89 micronuclei, where as the mean micronucleus count in "S" group was 5.36. The "A+ S" group had the mean micronucleus count of 5.98 and the control group had a mean micronucleus count of 3.67. *Conclusion:* The present study was undertaken to observe the micronucleus count and micronucleus index among smokers (S group), alcoholics (A group) and smokers and alcoholics together (A+S group) and compare with controls (C group) that had no such life style habits. In this study there was a significant difference between smokers and controls which seem to be more prominent in cases of both smoking and alcoholic group when compared to controls and the significance of alcohol only, was not well proven.

Keywords: Buccal Mucosa; Micronucleus.

Introduction

In the developing countries oral cavity is the fourth commonest site of carcinoma after lung, stomach and liver in males: while it is the fifth commonest cancer after cervix, breast, stomach and lung in females. Over the years the incidence of oral cancers in the population is increasing especially among younger generations possibly related to rising trends of pan masala, gutkha chewing, smoking, alcohol consumption [1].

Cytological study of oral mucosal cells is a non aggressive technique that is well accepted by the patients and has broad potential to fill the diagnostic gap that currently challenges the early detection of oral cancers and precancerous condition [2].

Epithelial tissue of the oral cavity is in immediate contact with ingested genotoxic agents and is accessible for sample collection. The epithelial cells can be easily collected from the mouth without causing discomfort to the patients. Assessment of micronuclei in epithelial cells of oral cavity is feasible, cheap and accurate procedure. Micronucleus has been used consistently as biomarkers for assessment of DNA damage [3].

The analysis of micronuclei has gained popularity as invitro genotoxicity test and as a biomarker assay for human genotoxic exposure and effect. The main reason for this development is in comparison with chromosomal aberrations the scoring of micronuclei is simple, requires less training and less time consuming. It is expected to be more sensitive than chromosomal aberration assay, because of the

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increased statistical power brought out by the fact that number of cells analyzed can easily be increased to thousands when only hundreds of cells are usually analyzed for chromosomal aberrations [4]. Hence, we have decided to use micro nuclear assessment for evaluating the genotoxic effects of alcohol & smoking separately as well as alcohol and smoking together.

Materials & Methods

Our study comprised of a total of 100 persons which were divided into four groups with 25 persons having alcohol consuming, 25 persons were tobacco consumers, 25 were both alcohol consumers and tobacco consumers and 25 formed control group who neither consumed alcohol nor tobacco.

Two cytological smears were taken from each person with the help of cytological brush. The smear was then immediately wet fixed in ethyl alcohol and then stained with routine Hematoxylin & Eosin staining technique and assessed for micronuclei count. 500 cells per slide were counted to note the changes at 400X magnification.

In the present study, criteria used for identification of micronuclei were suggested by Sarto et al [5]. Micronuclei were scored only when chromatin structure and color intensively were similar to or weaker than those of the main nucleus, borders were distinctly recognizable, they were round and were included within the same cytoplasm. Dead or degenerating cells were excluded from evaluation.

Method of Counting Cells

Zig zag method was used to count cells from each field. It is seen first horizontally and then vertically in a upward direction and then it is done in the same manner in downward direction. The whole slide is scanned in the same manner for the presence of micronucleus. The numbers of cells are counted by pressing the button on differential cell counter and if micronuclei are seen in any of the cell another button is pressed and the counts are calculated. The micronucleus index was calculated as

$$\text{Micronucleus index} = \frac{\text{Micronuclei count}}{\text{Number of cells screened from each slide}}$$

Statistical Analysis

On comparisons of each group with the control, the difference between "S" group and "A+S" group with the control was significant with a p value <0.05(P < 0.05). The difference between "A" group and control had shown a p value just above 0.05 (P>0.05).

Result

We have undertaken a case control study to observe and calculate micronuclei and micronucleus index in smokers and alcoholics with individuals with history of no smoking and non alcoholics. A three pronged approach to compare smokers (S group) with a control of non smokers and non alcoholics (C group), alcoholics group (A group) with same control group and smoker and alcoholic group (S+A group) with same control group.

In smokers group (S group) 32% of the cases were in the age group of both 41-50 years and 51-60 years. A 24% of cases in 31-40 years age group and 12% are in 21-30 years age group. Mean age of the group is 50.40 years with standard deviation of 13.66 years. When compared with control group (C group) it has 55.10 years mean age with standard deviation of 13.6 years, most cases were in 41-50 years age group at 40% and 32% of cases were in 51-60 years age group, with 16 % cases in 31-40 years age. A 4 % of cases were in 21-30 years group [Table 1].

We have found when age matched there is no significant difference in age of the both groups with a statistically insignificant difference of mean age 4.7 years.

In alcoholic group (A group) there were 32% cases were in 51-60 years age group and in 41-50 years age group 24% with 20% in 31-40 years age group. A 16% of cases were seen in 61-70 years age group with 8% of cases was in 21-30 years age group. The mean age for "A" group was 57.18 years with standard deviation of 12.61 years [Table 1].

When compared with control group there was no significant difference in age matching of "A" group with control group, with a minimal difference in mean age of 2.08 years.

When compared with mean age of control group, the alcoholic and smokers group had shown a mean age difference of 2.43 years, which was of good match without significant difference in age matching . The "A+S" group shown a 32% cases in 61-70 years age group and 24% of cases in 51-60 years age group . A 16% of cases were in 41-50 years age group with 8% each in 71-80 years and 21-30 years age group [Table 1].

In our study there is a good age matching between

the groups of cases without any significant statistical difference.

The micronucleus count of smokers is 40% of the cases showing 1-5 micronuclei and 32% showing 6-10 micronuclei and 16% showing more than 10 nuclei with a 12% showing no micronuclei. The control group had shown 56% of cases having 1-5 micronuclei and 32% cases had no micronuclei with a 8 %cases showing 6-10 micronuclei where as a 4% cases showed >10micronuclei [Table 2].

The mean micronucleus count for smokers group is 5.36±6.01. Control groups the mean micronucleus count is at 3.67±6.64. The difference is statistically with a P value less than 0.05 (P<0.05).

The alcohol only group ("A" group) had the micronucleus counts as 56% cases showing 1-5 micronuclei and 20% had not shown more than 10 micronuclei , 16% cases shown 6-10 micronuclei and a 8% cases had shown more than 10 micronuclei [Table 2].

The mean micronucleus count is at 4.98±5.90 compare to 3.67±6.67 of control group it does not show a good statistical significance with P value just above 0.05(P>0.05).

The smoker and alcohol group had shown a 6-10 micronuclei in 52% of cases and 24% cases showing 1-5 micronuclei, where as a 20% cases had more than 10 micronuclei. A 4% had shown no micronuclei. The mean micronucleus count for "S+A" group is 5.98±6.16. The comparison with control group had shown a P value of less than 0.05 indicating a statistical significance (P<0.05) [Table 2].

The comparison of micronucleus index also yielded similar result as micronucleus count with statistically significant correlation between control and "S "group and "S+A" group with a P value less than 0.05(P<0.05). But the comparison with alcohol ("A" group) group has yielded a P value of just above 0.05(P>0.05) [Table 3].

The comparison of mean micronucleus counts with control group clearly show the differences between each group with maximum difference is with "A+S" group followed by "s" group with a marginal difference with "A" group [Table 4].

Table 1: Age distribution of smokers, alcoholics, alcoholics and smokers with control group.

Age in years	Smokers (S group)		Alcoholic group (A group)		Alcoholic+Smokers group (A+S group)		Control group	
	No	%	No	%	No	%	No	%
21-30	3	12%	2	8%	2	8%	1	4%
31-40	6	24%	5	20%	3	12%	4	16%
41-50	8	32%	6	24%	4	16%	10	40%
51-60	7	28%	8	32%	6	24%	8	32%
61-70	1	4%	4	16%	8	32%	2	8%
71-80	-	-	-	-	2	8%	-	-
Mean ±SD	50.40±13.66		57.18±12.61		57.53±13.48		55.10±13.75	

Table 2: Comparison of micronucleus count between control and smokers, alcoholics, alcoholics and smokers

Micronucleus count	Smokers (S group)		Alcoholic group (A group)		Smokers +alcohol group (A+S group)		Control group	
	No	%	No	%	No	%	No	%
Nil	3	12%	5	20%	1	4%	8	32%
1-5	10	40%	14	56%	6	24%	14	56%
6-10	8	32%	4	16%	13	52%	2	8%
>10	4	16%	2	8%	5	20%	1	4%

Table 3: Comparison of micronucleus index between controls and smokers, alcoholics, alcoholics and smokers

Micronucleus index	Smokers (S group)		Alcoholic group (A group)		Smokers +alcohol group(A+S group)		Control group	
	No	%	No	%	No	%	No	%
Nil	3	12%	5	20%	1	4%	8	32%
<0.01	10	40%	14	56%	6	24%	14	56%
0.01-0.02	8	32%	4	16%	13	52%	2	8%
>0.2	4	16%	2	8%	5	20%	1	4%

Table 4: Comparison of mean mn count between all three groups with control group

Group	Mean MN count	Mean MN count of control ("C" group)
"S" group	5.36±6.01	3.67±6.64
"A" group	4.89±5.90	3.67±6.64
"S+A" group	5.98±6.16	3.67±6.64

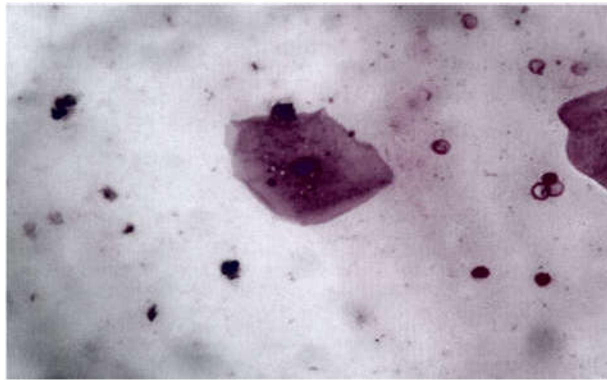

Fig. 1: Oral buccal mucosal cells having single micronucleus (H & E, X400)

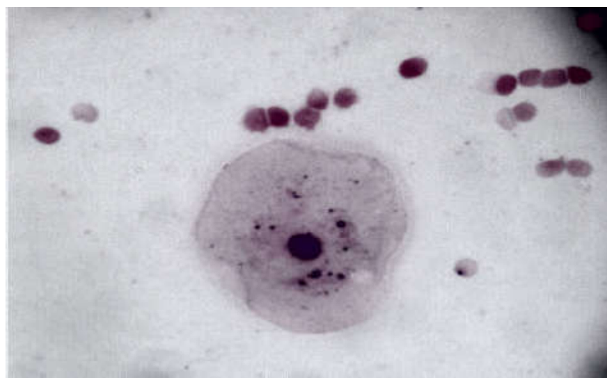

Fig. 2: Oral buccal mucosal cells having multiple micronuclei (H & E, X400)

Discussion

Exfoliative cells from oral epithelium have been widely used in cytology to detect abnormal nuclear and cellular morphology depicting precancerous and cancerous changes. Genetic changes in these cells are of particular interest [6]. Buccal mucosal cells are seen to be widely affected as more surface area of the buccal mucosa is exposed to the insult in the oral cavity and the fact that these epithelial cells are non-keratinized, makes them more vulnerable to change [7].

Biomarker is a measurable DNA and RNA characteristic that is used as an indicator of biologic and pathogenic process. The biomarkers can be translated into the relationship between exposure and disease and thus act as an indicator of the disease process [8]. In the present study, micronucleus count is a biomarker used to assess the proliferation potential. Micronucleus assay can be used to measure DNA damage in the proliferative cell as these arise from chromosomal fragment lagging behind during cell division, which appear as small dots with the similar intensity of the main nucleus. Others stains which can be used to identify the micronucleus in Acridane orange in which micronucleus appear as green dots in the yellow orange cytoplasm of an exfoliated cells [7]. In the present study, Hematoxylin & Eosin stain was used to calculate the micronucleus count and micronucleus Index. Micronucleus color intensity similar to main nucleus (Figure 1& 2).

Oral habits such as tobacco and alcohol consumption are said to be important etiologic factors for carcinogenic cytological change [6]. Around two-third of squamous cell carcinoma and 75% of head and neck cancer have been attributed to tobacco and alcohol consumption.

The analysis of cell proliferation and DNA damaged has gained popularity as an in vitro genotoxicity test. In the present study results showed that in case of tobacco consumer's proliferation potential in the form of DNA damage with a mean micronucleus count of 5.36 which is significantly higher than control group.

Genotoxic carcinogens, mainly N-nitrosonornicotine, polycyclic aromatic hydrocarbons present in the tobacco act on the keratinocytes and then enter the nucleus, where they are metabolized by cytochrome P 450 and glutathione S transferase.The electrophilic intermediate thus produced, binds with DNA by direct stimulation of heat of cigarette and chemical action of volatile products of tobacco causing altered cell proliferation and DNA damage. These events lead to unrepaired alterations or mutations in the DNA that may further progress to carcinogenesis [9]. This was in accordance with the present study as the DNA damage was increased in the tobacco consumers, which might cumulatively lead to carcinomatous changes. Bohrer et al in 2005 [10]. Assessed the presence of micronuclei in exfoliated oral mucosal cells collected from three anatomic sites in patients exposed to tobacco and alcohol and found a trend toward an increased number of micro nucleated cells in tobacco and alcohol user's at all anatomic sites.

Reis et al in 2002 [11]. did a study to assess the frequency of micronuclei in exfoliated cells from the tongue and buccal mucosa of alcoholic individuals

and found that frequency of micronuclei in buccal mucosa cells was higher in the group of alcoholic individuals, when compared to the control group, although the difference was not statistically significant (p>0.05). The results of these studies are consistent with the present study.

The synergistic effect of alcohol and tobacco showed a significantly higher proliferation index and DNA damage, when compared to either of the two substances alone. This was depicted by significantly higher micronuclei count in this group. Alcoholic beverages exert synergistic effect with tobacco [12]. In a similar study, Stich and Rosin 1983 [13]. Observed the effect of tobacco and alcohol on the exfoliated cell of buccal mucosa by using the micronucleus assay. They found a strong synergistic effect between smoking and alcohol consumption as seen by the elevated frequency of micro nucleated buccal mucosa cells. Alcoholic beverages contain alcohol, acetaldehyde and nitrosamine that alter the rate of penetration of substances from the oral environment across the mucosa that have a role in carcinogesis. Acetaldehyde produced by microflora by the oxidation of ethanol is predominantly responsible for alcohol associated carcinogenesis. It binds With the DNA and protein resulting in hyperproliferation.It is clear that oral cancer risk is related to both intensity and duration of alcohol and tobacco consumption. According to world Health Organization over 1 billion people are currently associated with tobacco smoking and nearly 2 billion adults worldwide are estimated to consume alcoholic beverages regularly, with average daily consumption of 13 g of ethanol (about one drink)(IARC Monographs 2009). Alcohol use (>5drinks/day) along with tobacco use (>20 cigarettes/day) increase the drink of oral cancer than expected based upon the independent effects of the same amount of alcohol or tobacco alone [14]. In addition, tobacco smoking is estimated to account for approximately 4-5 million deaths a year worldwide. This number is projected to increase to approximately 10 million a year by 2030 [15].

Conclusion

In the present study 100 cases were studied among which 25 were control group ("C" group) 25 were alcoholics cases ("A" group) 25 were smoker cases ("S" group) and 25 were alcohol+smoker cases ("A+S" group). A mean age in "A" group was 57.18 years, whereas the mean age in "S group" was 50.14 years, with a mean age of 57.53 years in "A+S group". The mean age was 55.10 years in control group. The micronucleus count in "A group" was a mean count of 4.89 micronuclei, where as the mean micronucleus count in "S group" was 5.36.The "A+S" group had shown a micronucleus count of 5.98. The controls had a micronucleus count of 3.67.

The micronucleus index also yielded similar results as micronucleus count. Validating the study statistics. The micronucleus count had indicated that smoking and smoking with alcohol has significant genotoxic effect on oral mucosal cells more so when smoking is combined with alcohol. Alcohol alone doesn't seem to have significant effect on micronucleus count in our study.

This study, thus, shows that tobacco and/or alcohol may cause alterations in the oral mucosal cells with alcohol causing less severe effects than tobacco and together, their synergistic effects cause even more severe changes at cellular levels, which may increase the chances of progression to oral cancer. This study supports and extends the view that cytological changes on oral mucosa due to tobacco and alcohol can serve as a useful diagnostic adjunct for the early detection of oral premalignant changes and cancer. Furthermore, these may also be used as an educational tool for population awareness programs to help in cessation of these oral habits.

Abbreviations

1. S group-Smoker group
2. A group-Alcohol group
3. A+S group-Alcoholics & Smoker group
4. C group-control group
5. H & E : Hematoxylin & Eosin

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