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Cytological evaluation of oral mucosa in habitual Pan Masala eaters- A comparative study

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Abstract: *Objectives:* The study was undertaken to evaluate cytological changes that occur in the oral mucosa of habitual Pan Masala eaters. *Background:* The present study was undertaken to find the various morphological changes that occur in the oral mucosa of habitual Pan masala eaters. *Methods:* Samples from 250 individuals who were eating pan masala for more than 6 months and 250 non-eaters by cotton tipped applicator by scraping the buccal mucosa. Smears were prepared and stained by papanicolou method Atleast 1000 cells were scanned per slide under high power. *Result:* Total 1,76,530 and 1,26,869 cells were counted in all Test slides and control slides respectively. Statistically high significant difference was found between user and control group. *Conclusion:* Finding are accumulating regarding the local genotoxic effect such as occurance of micronucleated cells, cells with multiple nuclei, cells with broken egg nuclei, binucleated, and hyperkeratotic cells. These cells were increased according to duration and frequency of pan masala eating. The significance of occurrence of these cells and development of oral cancers requires further studies. Can these parameters be used for early detection of oral cancers? This study might answer this question and may help in reducing the number of oral cancers.

Key words: Pan masala, genotoxic, micronucleated cells, oral cancer.

Introduction

In recent years, the habit of panmasala chewing is increasing, owing to the assumption that, it is safe alternative to tobacco chewing [1] which is a known carcinogen [2-3] and also owing to its social acceptance. Panmasala is a dry powered complex mixture of various constituents which include arecanut, catechu, lime, cardamom, menthol, sandal oil, spices and unspecified flavouring agent. Panmasala is available as i) Panmasala plain ii) Sweet panmasala iii) Panmasala with tobacco. The harmful effects of areca nut, catechu and lime have been well documented. The chemical analysis of the different brands of panmasala has shown the presence of polycyclic aromatic hydrocarbons, nitrosamines, toxic metals and residual pesticides which are known pro-carcinogens. Already incidence of oral cancer is increasing day by day with as many as 17% to 48% of all cancer found in oral cavity with such a major health problem already on hand, in recent years, the habit of panmasala eating (chewing) is increasing, owing to the assumption that it is safe alternative to tobacco chewing. Which is a known carcingoen and also owning to its social acceptance. The constitutent, of panmasala are having genotoxic effect, one of the cytogenetic end point of this effect is micronucleated cells.

For the assessment of the genotoxicity of various chemicals, the demonstration of chromosomal aberretion (CA) or sister chromatied exchange or percentage of micro nucleated cells, which are cytogenetic end points are used as markers. Out of these three markers, demonstration of the micronucleated cells does not require cell culture and the preparation of meatphase spreads. This phenomenon of micronuleus formation also has been studied on exfoliated human bucccal cells. The significance of micronuclei, binucleation, broken egg nuclei and hyperkeratotic cells and development of oral cancer requires further studies. Can these parameters be used for early detection of oral cancers? This study might answer this question and may help in reducing the number of oral cancer. With this background the present was undertaken to study the various morphological changes that occur in the oral

mucosa of habitual panmasala eaters. The samples were obtained by exfoliated cytology which is a rapid, non-invasive, inexpensive procedure.

Aims of Study:

1. To study the cytological changes that occur in the oral mucosa of habitual pan masala eaters.
2. To evaluate and correlate the cytological changes in view of duration and frequency of pan masala eating.

Material and Methods

Samples from the oral mucosa of 250 habitual pan masala eaters and 250 non-eaters were taken. Individual eating pan masala for more than 6 months of duration with same age and sex were taken as a subjects.

A cotton tipped applicator was used to take the samples by scraping the buccal mucosa by means of linear and rotational movements. Then smeared on to a clean oil free glass slide. Then fix in absolute alchol for 15 to 30mins and stained with papanicolau's method.

Results

Comments: The habit is more prevalent amongst the younger population aged 21-25 and 26-30.

Cellularity Statistics		
Parameters	**User Group**	**Control Group**
Total no. of cells counted	176530	126869
Nucleated cells	147625	99806
Hyperkeratotic cells	38905	27063
Micro nucleated cells	25562	10440

Nuclear Characteristics Observed		
Parameters	**User Group**	**Control Group**
Micronuclei	139873	61741
Broken egg nuclei	909	323
Binucleate cells	15755	9417

Table Showing Cellularity Between User Group With Lesions And Without Lesions		
Parameters	**User Group with lesion**	**User Group without lesion**
C M I	15368	10194
Total No micronuclei	77886	61987
Broken egg	81	828
Binucleate cells	4004	11751
Hyperkeratotic cells	22543	16362
Nucleated cells	51083	96542

Comments: In our study user group with lesion we found 58 cases out of 250 i.e., 192 cases user group without lesion.

Micronucleated Cells Statistics		
Parameters	**User Group**	**Control Group**
Total number of cells	25562	10440
C M i/100 cells	14.48%	8.2%
C M i/100 NC	17.31%	10.46%
User: Control (/ 100 NC)	1. 76: 1	
User: Control (/ 100 NC)	1. 65: 1	

Statistic Of Micronucleated Cells Between User And Control Group						
Parameters	**User GP**	**Range**	**Mean±SD**	**Control GP**	**Range**	**Mean±SD**
Total no. of cells	25562	22 – 121	5±21.4102.25	10440	23 – 63	41.76±8.66

Test Statistics P value
Z = 14.03 P < 0.01 HS*
HS* = Highly significant SD = Standard Deviation

A highly significant difference was found the User group and Control group.

Micronucleated Cells Statistics Between User Group With Lesions And Without Lesions			
Parameters	**Control GP 250**	**User GP with lesions N=58**	**User GP without lesions N=192**
Total number of CMi	10440	15368	10194
C M i/ 100 cells	8.2%	8.70%	5.77%
C M i/ 100 NC	10.46%	10.41%	6.90%
Lesion: No lesion (/ 100 cells)	1. 50 : 1		
Lesion: No lesion (/ 100 NC)	1. 50 : 1		
No lesion: Control (/ 100 cells)	1. 42 : 1		
No lesion: Control (/ 100 NC)	1. 5 : 1		

Statistic Of Micronucleated Cells Bewteen User And Control Group						
Parameters	**User GP**	**Range**	**Mean±SD**	**Control GP**	**Range**	**Mean±SD**
Total no. of CMi	15368	18-700	264.97±51.72	10194	59-125	53.09±13.43

Z = 6.69 P < 0.01 HS*
Highly significant difference was found between user group with lesion and Control group at P = < 0.01.

Micronucleus Statistics Between User And Control Group		
Parameters	**User Group**	**Control Group**
Total number of cells	139873	61741
M i/1000 cells	792.3	486.65
M i/1000 NC	947.4	618.6
User: Control ratio (/ 1000 NC)	1. 62 : 1	
User: Control ratio (/ 10O0 NC)	1. 53: 1	

Statistics Of Micronucleus Between User And Control Group						
Parameters	**User GP**	**Range**	**Mean±SD**	**Control GP**	**Range**	**Mean±SD**
Total no. of micronuclei	139873	26-906	559.49±146.68	61741	73-400	246.96±47

Z = 15.66, P = < 0.01 HS*
A highly significant difference was found between user group at P = < 0.01.

Table Showing The Comparative Micronuclei Statistics Betwwen The User Group With Lesions And Without Lesions			
Parameters	**Control GP 250**	**User GP with lesions N=68**	**User GP without lesions N=192**
Total no. of micronuclei	61741	61987	77886
M i/ 100 cells	486.6	351.1	441
M i/ 100 NC	618.6	419.8	527.5
Lesion: No lesion (/ 1000 cells)	1. 25 : 1		
Lesion: No lesion (/ 1000 NC)	1. 25 : 1		
No lesion: Control (/ 1000 cells)	1. 1 : 38		
No lesion: Control (/ 1000 NC)	1 : 1. 47		

Broken Egg Nucleus Between User And Control Group		
Parameters	**User Group**	**Control Group**
Total number B-egg	909	323
B-egg/1000 cells	5.14	2.54
B-egg/1000 NC	6.15	3.2
User: Control ratio (/ 1000 NC)	2. 02 : 1	
User: Control ratio (/ 10O0 NC)	1. 91 : 1	

Statistic Of Broken Egg Nucleus Betwwen User And Control Group						
Parameters	**User GP**	**Range**	**Mean±SD**	**Control GP**	**Range**	**Mean±SD**
Total no. of B-egg	909	0.14	3.04±2.91	323	0 – 7	1.3±1.398

Z = 11.9, P = < 0.01 HS* A highly significant difference between user group.

A Comparative Broken Egg Nuclei Statistics Between The User Group With Lesions And Without Lesions			
Parameters	**Control GP 250**	**User GP with lesions N=68**	**User GP without lesions N=192**
Total no. of B-egg	323	81	828
B-egg / 1000 cells	2.54	0.68	4.69
B-egg / 1000 NC	3.2	0.81	5.6
Lesion: No lesion (/ 1000 cells)	1 : 6. 9		
Lesion: No lesion (/ 1000 cells)	1 : 6. 9		
No lesion: Control (/ 1000 NC)	1. 85 : 1		

Statistics Of Broken Egg Nuclei Between User Group And User Group Without Lesion						
Parameters	**User GP with lesion**	**Range**	**Mean±SD**	**User GP without lesion**	**Range**	**Mean±SD**
Total no of B-egg	81	0-3	0.324+0.57	828	0-21	3.31+4.67

Z + 10.05
No significant difference at P + <0.01 between user group with lesion and user group without lesions.

Binucleate Cells Between User And Control Group		
Parameters	**User Group N1 +250**	**Control Group N2 + 250**
Total no. of Bin	15755	9417
Bin / 1000 cells	89.2	74.2
Bin / 1000 NC	106.7	94.3
User : Control (/ 1000 cells)	1.2.:1	
User : Control (/ 1000 NC)	1.1:1	

Statistics Of Binucleate Cells Between User And Control Group						
Parameters	**User GP**	**Range**	**Mean±SD**	**Control GP**	**Range**	**Mean±SD**
Total no of Bin	15755	40-90	63.02+8.7	9417	30-64	37.7+9.4

Z + 25.35, P <0.01 HS*

Comparative Binucleate Cells Statistics Betweet The User Group With Lesions And Without Lesions			
Parameters	**Control GP**	**User GP WITH LESION n1+58**	**User GP without lesion n2+192**
Total no. of Bin	9417	4004	11771
Bin / 1000 cells	74.2	22.68	66.67
Bin / 1000 NC	94.3	27.12	79.73
Lession: No lesion (/ 1000 cells)	0.34:1		
Lession: No lesion (/ 1000 NC)	0.34:1		
Without lesion : control (/ 1000 Cells)	0.84		
Without lesion : control (/ 1000 NC)	0.84		

Statistics Of Binucleate Cells Between User Group With Lesions And Without Lesions						
Parameters	**User GP Without lesion**	**Range**	**Mean±SD**	**User GP with lesion**	**Range**	**Mean±SD**
Total no of Bin	4004	41-62	69.03+13.52	11771	40-72	61.2+6.52

Z + 7.83 +4.36
There is no significant difference at P= <0.01 between user group with lesions and without lesions.

Nucleated Cells, Hyperkeratotic Cells		
Parameters	**User Group N1 +250**	**Control Group N2 + 250**
No. of H cells	38905	27063
No. of NC cells	147625	99806
NC : H	3.79:1	3.6:1
% of H Cells	22.04%	21.3%

Statistics Of Nucleated And Hyperkeratotic Cells						
Parameters	**User GP N1=250**	**Range**	**Mean±SD**	**Control GP N2=250**	**Range**	**Mean±SD**
No of H cells	38905	65-221	155.62+29.69	27063	82-199	108.3+21.93
No of NC cells	147625	219-934	590.5+179.1	99806	48-769	399.0+124.05

1. No. of H cells – Z = 3.16 P<0.01 HS*
2. No. of N cells – Control S user group Z + 13.88 P<0.01 HS*

Nucleated Cells, Hyperkeratotic Cells Between User Group With Lesion And Without Lesions			
Parameters	**Control GP**	**User GP with lesions N1=58**	**User GP without lesions N1=58**
No. of H cells	27063	22543	16362
No. of NC	99806	51083	96542
% of H cells	21.3%	12.77%	9.27%
NC: H Cells	3.6:1	2.27	5.90

Statistics Of Nucleated Cells And Hyperkeratotic Cells Beween User Group With Lesions And Without Lesions						
Parameters	**User GP Without lesion N1-58**	**Range**	**Mean±SD**	**User GP without lesion N2-192**	**Range**	**Mean±SD**
No of H cells	22543	39-710	388.67+66.24	16362	32-201	85.22+32.99
No of NC cells	96542	107-775	502.8+123	51083	689-810	880.7+71.03

1. No. of H cells – Z = 2.60 Significant at = 0.05
2. No. of N cells – Z = 29.35 P < 0.05 HS*

Fig-1: Microphotograph showing increased number of hyperkeratotic cells (Pap x 200)

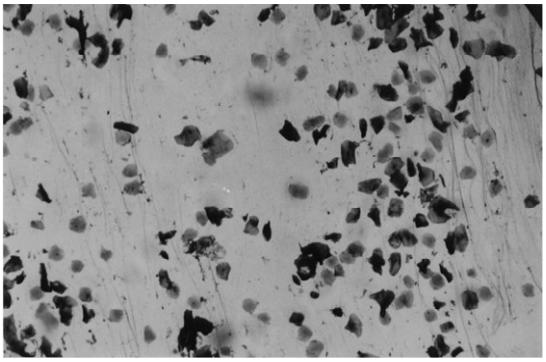


Fig-2: Microphotograph showing cells with multiple micro nuclei (Pap x 1000)

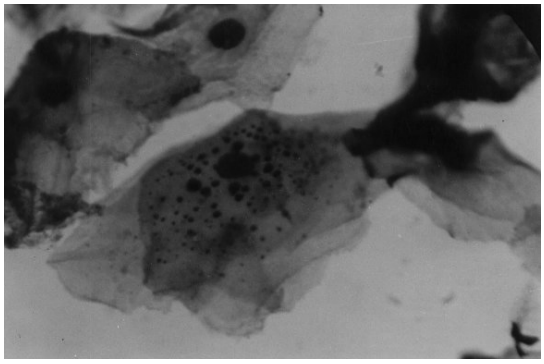


Fig-3: Micrograph showing with broken egg with nuclei (Pap x 1000)

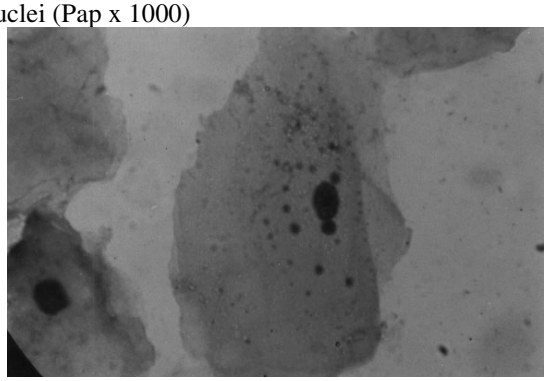
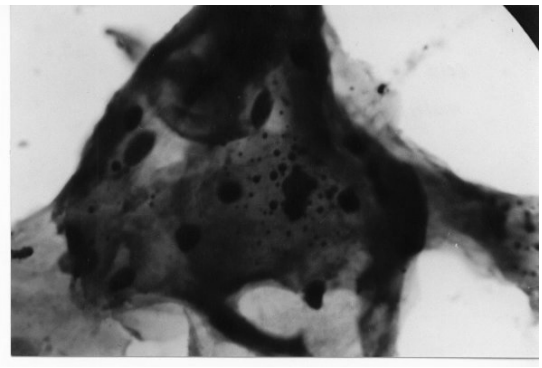


Fig-4: Binucleated cell with multiple micro nuclei (Pap x 1000)



Correlation Between The Average Number Of Packets Consumed Per Day And Occurrence Of Micronucleated Cells		
Duration in year	**Packets per day**	**Micronucleated cells**
½ - 5 years	2 – 6 per day	3573
6 – 10 years	6 - 10 per day	9789
11 – 15 years	10 – 14 per day	12200

Correlation test applied to the occurance of micronuleated cells in relation to average number of packets consumed per day in three different duration group (1/2 – 5 Year, 6 – 10 years and 11 – 14 years).

According to duration and frequency of consumption of panmasala increase the number of micronucleated cells. This was consisted with Ghosh et.al [4] who found in their study increased frequency of micronuleated cells to be higher in user group. In 1991 Mukherjee et.al [5] observed that there was high frequency of microuncleated

cells among Indians chewing betel quid, arecanut and tobacco. In our study we found that there was increased number of micronucleated cells in user group as compared to the control group.

Discussion

Findings are accumulating the local gentoxic effects of various "chewable substances" like tobacco, arecanut, catechu, lime etc by various independent studies. Panmasala being a complex mixture of all these ingredients and more, poses a problem in this regard since all these various components can have an antagonistic or synergistic effect. Through we did find several invitro studies demonstrating the genotoxic and the clastogenic effect of panmasala extract, we found that very few in vitro studies have be done directly assess its local gentoxicity. With this background the study was taken to evaluate the cytological changes in the oral mucosa of habitual panmasala eaters. In our present study, the cytological changes that were examined as markers were the occurrence of micronuclei, broken-eggnuclei binucleated cells and hyperkeratotic cells.

Micro-Nuclei: Minronuclei are considered to be markers of abnormal mitosis. This involves chromosomal breakage and misaggregated chromatin, which results in the formation of a separate smaller nucleus. We found a few studies were done in this direction. In 1996 Trivedi et.al [2,6] have reported a significantly higher frequency of micronuleated cells in exfoliated buccal mucosa in users of both plain panmasala and panmasala with tobacco when compared with control population. Our study was consistent with this finding since it was seen that 8.8% of the cells were micronucleated in the user group, only 8.2% were micronucleated in the controls. The CMI ratio of user; control thus up to 1.07:1. These values however are very high when compared to the result of a study by Stich et.al [7] who found 0.47% of buccal mucosal cells contained micronuclei in the control Indian population. And that this level is increased to 2.2% of even 8.4% in betel quid tobacco chewers, but the background levels of micronuclei in exfoliated buccal cells reported in the literature vary between 0.03 and 0.47%16, more than a ten fold variation.

The high variability in background levels reported may be several factors including.

1. Scoring criteria
2. Staining intensity
3. Number of cells scrored per individual

Nevertheless a very high significant difference existed between the number of micronucleated cells amongst user and control group at $P<0.01$. The percentage of micronucleated cells were found to be much higher among the user group than control group. This finding is consistent with the finding of Patel et.al [1]. which states that ethanol potentiates the gentoxic effects of panmasala. The finding further led to its conclusion that smoking alcohal and tobacco in any other form potentiates the genotoxic effect of panmasala on the buccal mucosal cells. The percentage of micronuleated cells increased in the presence of oral lesions which may suggest a distinct inflammatory pathology.

The user group also showed an increased incidence of hyperkeratotic cells and this might have brought about the disparity in the ratio between the number of micronuclei in user and control group. Nevertheless even the ration of number of micronuclei per 1000 nucleated cells in both user and control groups showed significant 1.53:1.

Broken egg nuclei: The phenomenon of "broken egg nuclei" was described by Tolbert and co-workers [8] in 1992. Typically these are cells containing unequal sized nuclei connected by a thin bridge of Feulgen-positive material. These could be related to anaphase bridges, which arise as a result of chromosome aberrations and failure to complete mitosis. However the precise origin and significance of this very abnormal nuclear event is still unknown. Nina Titenko et.al [9] reported the average frequency of "broken eggs" to be 0.05 + 0.04% in control population. The value we found i.e. 2.54% in control group the value rose to 5.14% in the user group. Through there was a definite increase in the number of B-eggs. There was highly significance between user group and control group at $P<0.01$.

Binucleated cells: In 1992 this cell abnormality was recognized by Tolbert et.al. the cells in this category had two nuclei of similar size within the cytoplasm and could be a result of incomplete cell division. In 1994 Nina Titenko et.al [9] reported their average frequency to be 0.4+ 0.2.1% in the control subjects. The frequency of binucleates amongst the control group in our study was found to be 74.2 per 1000 cells and. The figure rose to 89.2 amongst the user group and showed a significant different at $P<0.01$. no correlation could be found between the binucleate cells and presence of oral lesions. Further studies needed to confirm significant of binucleate cells.

Hyperkeratotic cells: These are cells with a ghost nuclei or no visible nuclei and orangeophillic or eosinophillic cytoplasm. Occasionally the keratinisation may be very dense and refractile. Anderson et.al [10] reported increased number of mitotic figures above the basal layer, commonly found under hyperkeratotic lesion and a significant correlation between the anuleated cell incidence. In our study there were increased number of anucleated cells (H) in user group as compared to the control group. However we were unable to find any study correlating the incidence of hyperkeratotic cells with genotoxicity and this angle needs to be investigated further. Other nuclear changes like karyorrhexis, bare nuclei karyolysis and pyknosis were observed but not guantified.

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