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Assessment of Genotoxic Effects of Various Forms of Smokeless Tobacco Using Micronuclei Assay

[1] Ansu Ann Abraham, [*2] Ceena Denny E., [3] Nidhi Manaktala, [3] Srikant Natarajan, [2] Junaid Ahmed and [1] Utkarsh Yadav

[1] Manipal College of Dental Sciences, Manipal Academy of Higher Education, Mangalore, Karnataka, India, [2] Department of Oral Medicine and Radiology, Manipal College of Dental Sciences, Manipal Academy of Higher Education, Mangalore, Karnataka, India and [3] Department of Oral and Maxillofacial Pathology, Manipal College of Dental Sciences, Manipal Academy of Higher Education, Mangalore, Karnataka, India

Abstract

Aim: To assess the micronuclei assay based on age and gender variability from the buccal mucosal cells in patients using various forms of smokeless tobacco.

Materials and Methods: A cross sectional study was conducted among sixty eight individuals who had a history of chewing various forms of smokeless tobacco. The study subjects were divided according to their habits into four groups. The groups were arecanut, betel quid, gutkha and control group. Buccal smears were obtained and the exfoliated cells were stained using Feulgen stain. The micronuclei count was done under 40X magnification and compared among the various groups.

Results: One way ANOVA analysis was done to compare the difference in ages of the various groups in the present study. The mean age in years were: control group (21 years), gutkha (30 years), betel quid (50 years) and arecanut chewers (43 years), which was found to be significant ($p < 0.01$). One way ANOVA analysis was also done to compare the micronuclei values in the various groups and it was found that it was the highest in betel quid chewers ($P < 0.05$). The comparison of the micronuclei value between control and each of the habits was found to be significant ($p < 0.001$). Similarly, while comparing the micronuclei value between betel quid and the arecanut was found to be significant ($p = 0.003$).

Conclusion: The frequency of Micronuclei assay formation could be used as markers for early identification of the genotoxic effects of chewing tobacco.

Policy implications: Awareness of the toxic effects should be carried out with these target groups in mind.

Keywords: Carcinogen, feulgen stain, genotoxic effects, habits, micronuclei assay, mucosa, smokeless tobacco.

[*] Corresponding author: Department of Oral Medicine and Radiology, Manipal College of Dental Sciences, Manipal Academy of Higher Education, Mangalore, Karnataka, India.

Introduction

Cancer is one of the leading causes of death worldwide. It is known to be caused by a variety of genetic alterations. Due to the increased popularity of addictive habits in India, oral cancer is highly prevalent and accounts for 30-40 % of all the malignant lesions in the country. About 65,000 new oral and pharyngeal cancers are diagnosed annually in India. Among the sites of occurrence of oral cancer, buccal mucosa has been found to be the commonest. It has been observed that exposure to various environmental factors (known as carcinogens) causes a predilection for the incidence of cancer. One of these carcinogens is tobacco. People who consume alcohol and use tobacco products are at a higher risk to develop oral cancer. Tobacco causes the release of xenobiotic metabolizing enzymes which result in DNA damage. The body tries to repair the damage caused by this DNA damage and the inefficient repair leads to an increased risk of cancer. The WHO IARC Monographs Programme reported that betel-quid and areca-nut chewing is carcinogenic to humans, even when it is chewed without tobacco. They also reported that betel quid without tobacco is now known to cause oral cancer in humans. Areca nut, a common component of all betel quid preparations was also observed to cause oral sub mucous fibrosis (a pre-cancerous condition that can progress to malignant oral cancer), leading to the determination that areca nut itself is carcinogenic to humans. Various diagnostic tests for early detection of cancer are employed and they include micronuclei assay, brush biopsy, toluidine blue staining, auto-fluorescence, chemiluminescence, salivary proteomics, DNA analysis, biomarkers and spectroscopy etc. Among the various diagnostic tests, the micronuclei assay which is estimated from exfoliated buccal mucosal cells, is minimally invasive, very economic and causes minimum stress in the study subjects (Thomas *et al.,* 2008). The technique involves examination of epithelial smears to determine the prevalence of cells containing micronuclei which are extra nuclear bodies composed of chromosomes or chromosomal fragments that failed to be incorporated into daughter nuclei at mitosis. The various methods to detect micronuclei are staining with Feulgen stain, Acridine orange fluorescent stain, fluorescence in situ hybridization (FISH) with a centromeric probe and Giemsastain. The most widely used procedure for staining epithelial cell preparations for MN analysis involves a Feulgen reaction to identify the DNA of the nucleus and micronucleus, followed by a counterstain with Fast Green or Light Green to delineate cell cytoplasm. Micronucleus test is a good indicator of mitotic interference and also shows mutations or breakage of chromosomes. The test has been found to be 95% accurate and has a sensitivity of 94% and is 100% specific. Micronucleus (MN) is an extra small nucleus separated from the principal nucleus, generated during cellular division by late chromosomes or by chromosome fragments. Microscopically, it appears as oval to round cytoplasmic chromatin mass in the extra nuclear vicinity. They are induced in the cells by genotoxic agents that damage the chromosomes. The damaged chromosomes, in the form of acentric chromatids or chromosome fragments, lag behind in anaphase when centric elements move towards the spindle poles. After telophase, the undamaged chromosomes, as well as the centric fragments, give rise to regular daughter nuclei. The lagging elements are included in the daughter cells and a considerable number are transformed into one or several secondary nuclei, which are much smaller than the principal nucleus and known as micronuclei. It is known that consumption of tobacco in various commercial forms along with alcohol has a synergistic effect in causing oral cancer. Micronuclei assay is a reliable marker to study the genotoxic effects of various tobacco products on the oro-mucosal cells. Hence, this study was done to compare the micronuclei assay based on age and gender variability in various forms of smokeless tobacco (arecanut, betel quid, gutkha) products.

Materials and Methods

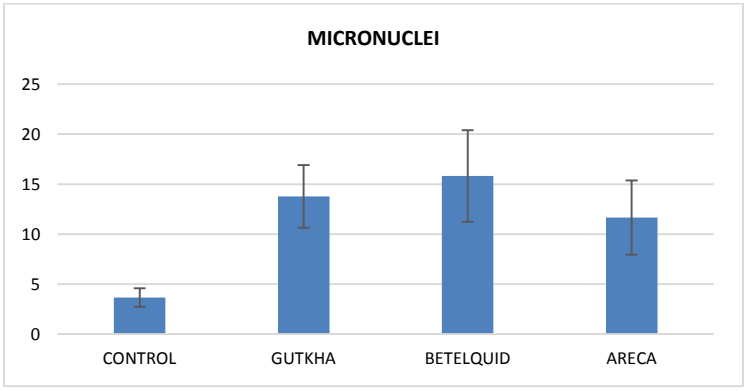
After obtaining ethical clearance for the study, patient information regarding age, sex, systemic conditions, duration and frequency of chewing, type and site of placement of the product was collected. Patient was asked to rinse his/her mouth before the smear was taken. Buccal smears were taken using a wooden spatula and the exfoliated cells were spread over clean glass slides and stained according to the method followed by Naderi *et al*. (2012). Prepared smears were fixed in cytological fixative or Carnoy's fixative (methanol and glacial acetic acid in a ratio of 3: 1. They were then rehydrated through descending grades of alcohol and brought to water. The smears were then rinsed in 1 M Hydrochloric acid at room temperature for 2-3 minutes. The smears were then placed in 1 M Hydrochloric acid solution of 55-60 °C for 10 minutes. This is to ensure sufficient time for acid hydrolysis. The smears were then rinsed in 1 M Hydrochloric acid solution at room temperature for 5 minutes followed by placement in distilled water. Schiff's reagent was then added to the slides following acid hydrolysis and kept for 45 minutes. After rinsing the Schiff's reagent, Haematoxylin was added and kept for 8 minutes. The slides were then dried for 2-3 minutes. The slides were then counterstained with light green for 1 minute. The smears were then dehydrated through ascending grades of alcohol to xylene and then mounted using DPX mounting media with a thin cover slip. Feulgen reaction following the modified method of Thomas *et al*., (2009) was used for determination of micronuclei. After the staining procedure, slides were mounted using DPX mounting media with a thin cover slip and approximately 500 cells were examined under the 40X magnification (Fig 1).

Figure 1: Photomicrograph showing epithelial cells stained with Feulgen (X 100) with a single cell [inset] exhibiting three micronuclei (X 1000 magnification)

Data management and statistical methods: Data was then analyzed using Statistical Package for Social Sciences (SPSS), version 20.0 (SPSS Inc., Chicago IL). Chi square and Fisher's exact tests were used for comparison of categorical data.

Results

Among the sixty eight subjects who participated in the study thirty one were males (45.58 %) and 37 subjects were females (54.42 %). The subjects were divided into four groups according to their habit, group 1 (control) group 2 (gutkha) group 3 (betel quid) and group 4 (arecanut), with 17 study subjects in each group. The subjects were of the age group 18 – 80 years with the mean age being 34.53 years (Table 1). The habits were cross-tabulated with the gender of the study subjects. Overall, subjects who chewed gutkha were predominantly males, while those who chewed betel quid and arecanut were predominantly females (Graph 1).



Graph 1: Comparison of micronuclei of the habits with the control shows there is a significant higher value of micronuclei compared to controls. With a p value of 42.485 and <0.001 the significance is between control and three groups and areca and betel quid. gutkha and betel quid had similar values of micronuclei presence.

Table 1: Comparison of habit distribution with gender

GROUP * Sex Cross tabulation

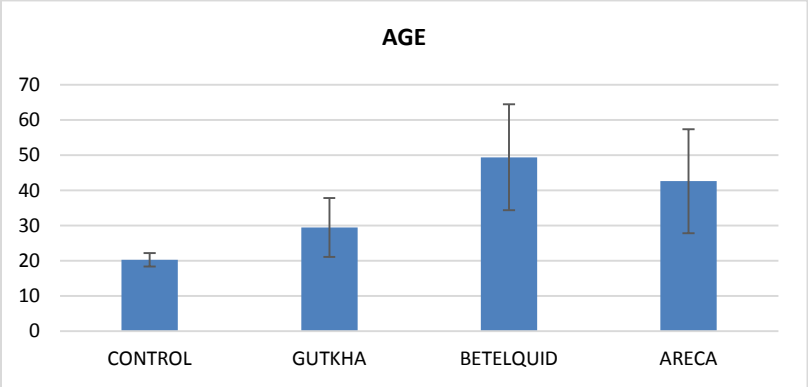
			Sex		Total
			F	M	
GROUP	CONTROL	Count	15	2	17
		% within Sex	40.5 %	6.5 %	25.0 %
	GUTKHA	Count	1	16	17
		% within Sex	2.7 %	51.6 %	25.0 %
	BETELQUID	Count	11	6	17
		% within Sex	29.7 %	19.4 %	25.0 %
	ARECA	Count	10	7	17
		% within Sex	27.0 %	22.6 %	25.0 %
Total		Count	37	31	68
		% within Sex	100.0 %	100.0 %	100.0 %

Chi square value of 24.840 and p value of <0.001

One way ANOVA analysis was done to compare the difference in ages of the various groups in the present study. The mean age in years was: control group 21 years, gutkha 30 years, betel quid 50 years and arecanut group was 43 years, which were found to be significant (p< 0.01). Also, the mean age of the total study subjects was found to be c approximately 35 years. From the results, it can be deduced that older individuals were more likely to have habits. (Table 2, Graph 2).

Table 2: Intergroup comparison of micronuclei

Dependent Variable	(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	P VALUE
Micronuclei	Control	Gutkha	-10.118*	1.156	<0.001
		BETELQUID	-12.176*	1.156	<0.001
		ARECA	-8.000*	1.156	<0.001
	Gutkha	Betelquid	-2.059	1.156	0.292
		ARECA	2.118	1.156	0.268
	Betelquid	Areca	4.176*	1.156	0.003
Age	Control	Gutkha	-9.176	3.761	0.081
		BETELQUID	-29.106*	3.884	<0.001
		ARECA	-22.321*	4.04	<0.001
	Gutkha	Betelquid	-19.929*	3.884	<0.001
		ARECA	-13.145*	4.04	0.01
	Betelquid	Areca	6.785	4.155	0.369



Graph 2: Comparison of the mean age of the study subjects in control, gutkha, betel quid and areca groups

One way ANOVA analysis was also done to compare the micronuclei values in the various groups. The mean value of micronucleus frequency detected from the buccal mucosa of the study subject's not chewing tobacco was 3.65, while the values in the subjects chewing gutkha, betel quid and arecanut were 13.76, 15.82 and 11.65 respectively. The mean micronuclei count was significantly higher in the subjects chewing tobacco ($P< 0.05$) than in the samples of subjects who did not chew tobacco. (Table 3, Graph 3). Comparison of the micronuclei count between individual groups with a dependent variable was done using Posthoc Tukey HSD analysis. The comparison of the micronuclei value between control and each of the habits was found to be significant ($p< 0.001$). However, the comparison of the value between gutkha and betel quid, and gutkha and arecanut was not found to be significant ($p= 0.292$ and $p= 0.268$ respectively). Similarly while comparing the micronuclei value between betel quid and the arecanut was found to be significant ($p= 0.003$) (Table 3). Posthoc Tukey HSD analysis was also done to compare the mean ages between individual groups with a dependent variable. Comparison of mean ages between control and betel quid, and control and arecanut were found to be significant ($p< 0.001$). Comparison of age between control and gutkha was not found to be significant ($p= 0.081$). Comparison of age between gutkha and betel quid ($p< 0.001$), and gutkha and arecanut ($p= 0.01$) were found to be significant. However, comparison of age between betel quid and the areca nut was not found to be significant ($p= 0.003$). The Pearson Chi-Square tests were also done to associate gender and the habits. They revealed a significant difference ($p<0.001$) (Table 4).

Discussion

Oral cancer is one of the leading causes for mortality and morbidity in our country. Various deleterious habits in the smokeless forms pose a threat to the humans all over the world and are seen to be increasing in the developing countries. In spite of this knowledge, the prevalence of consumption of tobacco and its related products is on the rise. Hence, early diagnosis helps us to detect the early changes occurring in the oro-mucosal cells. The use of a biomarker in disease development reflects the importance of the marker as a crucial indicator of a relationship between exposure and disease. Micronuclei assay is the simplest, most economical and minimally invasive method that helps us to measure the Deoxyribonucleic acid (DNA) damage more easily than the chromosomal aberrations. It does not require any specific DNA stain, most sensitive when compared to other tests and also does not require tedious procedures like cell culture and metaphase preparation. The present study was done to assess the micronuclei from the buccal mucosal cells in patients who chewed various forms of smokeless tobacco like gutkha, betel quid and arecanut. In our study, it was observed that the mean age of the subjects who chewed gutkha was 30 years which is in accordance to the study done by S Jyoti

et al., (2013). Betel quid users had a mean age of 50 years which is in accordance to the IARC monographs (2012) and Meerjady S Flora *et al*., (2012). The mean ages of arecanut chewers was 43 years which is similar to the study done by Chun-Nan Hsiao *et al*., (2015). Our study also showed a female predominance in consuming smokeless form of tobacco especially betel quid and arecanut which is similar to the study done by Singla *et al*., (2014) and Chun-Nan Hsiao (2015) respectively. This could be because females prefer self-prepared traditional tobacco chewing. According to Nayak (2011), more males were found to be using gutkha in comparison to females which was similar to our findings. The present study also shows that the mean number of micronuclei in buccal mucosa cells of the subjects who chewed tobacco is higher than that in the control group. The highest micronuclei values were found in betel quid, followed by gutkha, and areca nut and the least was observed in the control group. The higher micronuclei values in subjects who chew tobacco is in accordance with the studies by Ozkul *et al.,* (1997), Bansal *et al.,* (2012), Pradeep *et al.,* (2014), Sellappa *et al.,* (2009), Motgi *et al.,* (2014). In the present study, the mean number of micronuclei was highest in betel quid. The values in betel quid users were found to be about five-fold higher than the values in the control group. This result is higher than that observed by Faldu Hari Krishna. G. *et al*., (2011) who found a two-fold higher value in the micronuclei count between smokeless tobacco users and the control group. According to the WHO Monographs, 'betel quid' generally contains betel leaf, areca nut and slaked lime and may contain tobacco. Betel leaf and arecanut which are both known for their genotoxic effects, could be responsible for the higher micronuclei values in betel quid compared to the other forms of smokeless tobacco in the present study. This could be due to the synergistic effect of both betel leaf and areca nut. The mean micronuclei count in gutkha chewers was similar to a study done by S Jyoti *et al*., (2013). Arecanut was found to have a three-fold increased value when compared to the control group which was similar to the findings in the study conducted by Dave *et al.,* (1992). It has been suggested that the micronuclei in buccal mucosa may predict cancer risk for the upper aerodigestive tract, including premalignant stages such as oral leukoplakia. According to Winn *et al.,* (1981), the prime suspect for the constituent that is responsible for oral cancer is N′-nitrosonornicotine (NNN). NNN is derived through the action of salivary nitrates which generate high levels in subjects who place smokeless tobacco in the oral cavity for prolonged periods of time. This could be the reason for the higher values of micronuclei in smokeless tobacco users in comparison to the control group. According to Main *et al*, betel leaf (Piper betle) and sliced areca nut (Areca catechu) and/or powdered slaked lime are additives in smokeless tobacco that enhance the toxicity as well as the psychotropic effect of tobacco. Stich *et al*., (1984) established that an increase in micronuclei in exfoliated human cells predicts early risk of cancer and the increase in the count among smokeless tobacco users (betel quid, gutkha and arecanut) identifies the same. The increased micronuclei count in smokeless tobacco chewers could be due to the genotoxic effects of the constituents found in it. This has to be confirmed by studies in the future to provide more conclusive and reliable results. The development or progression of disease is influenced by various factors like genetic and dietary factors and susceptibility factors like carcinogen metabolism, apoptopic pathway genes and alteration in DNA repair. One of the main limitations of our study was that the sample size was less. In order to overcome this, the sample size can be increased to get more accurate results. Also, there is a possibility of misinterpretation of nuclear anomalies (e.g. karyorrhexis, karyolysis, condensed chromatin, and binucleates) and keratin granules as micronuclei.

Conclusion

The present study shows that consumption of smokeless tobacco is genotoxic and increases the risk of cancer. Micronuclei assay is a minimally invasive, inexpensive and a highly sensitive test which can be used in early detection and screening of individuals.

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