Original Article

Detection of Micronuclei in the Buccal mucosa of Arecanut and Gutka Chewers-An Indicator of Oral Cancer

Dr. Apeksha Mainali [1], Dr. Sumanth KN [2] Dr.Ravikiran Ongole [3], Dr. NIshant[4], Dr. Vishwas Sarlaya [5], Dr.Karen Boaz[6]

[1]Assistant Professor and Head, [2,4,5] Professor and Head, [3]Professor,[1,2,3] Department of Oral medicine and Radiology,[4] Department of Microbiology,[5] Department of Oral Pathology
[1]Nepal Medical College
[2]Manipal College of Dental Sciences, Melaka,Malaysia
[3,4,5]Manipal College of Dental Sciences, Kasturba Medical College, Mangalore

ABSTRACT

Introduction: The chewing of Arecanut is a common habit amongst all sections of society in South East Asia. Arecanut and commercially available products like *Gutka* contain genotoxic components that result in damage to cells leading to oral cancer. The frequency of occurrence of micronuclei has been used as an important dosimeter for assessing the genotoxic effects of chemical mutagens.
Objectives: The objective of the study was to assess the genotoxic effects of arecanut and *Gutka*: and to quantify the number of micronuclei in buccal mucosa of arecanut and *Gutka* chewers.
Method: The study was conducted in Manipal College of Dental Sciences, Mangalore, India. The study consisted of 140 individuals which included 3 groups. Group I was the control group that included 70 healthy individuals. Group II (subject) were arecanut chewers and Group III (subject) were *Gutka* chewers, with 35 individuals in each group. In the present study, the micronucleus test was applied to all 140 individuals.
Results: Out of the two varieties of arecanut, 80% were red variety and the rest 20% were white variety of arecanut. The results of this study showed that there was a significant elevation in micronucleated cells from the exfoliated oral mucosal cells obtained from arecanut chewers and *Gutka* chewers over control samples.
Conclusion: The increase in the number of micronucleated cells observed in chewers reinforced the possible genotoxic damage in chewers.

INTRODUCTION

The chewing of Arecanut (*Areca catechu*), popularly known as betel nut or *Supari* is a very common habit in South East Asia.[1] As an essential requisite for several religious and social ceremonies in South East Asia, arecanut (both red and white varieties) is extensively used by all sections of society.

Arecanut and commercially available products like *Gutka* contain genotoxic components that result in damage to cells.[2] The frequency of occurrence of micronuclei has been used as an important dosimeter and sensitive parameter for assessing the genotoxic effects of chemical mutagens.[3]

In human carcinogenic processes, micronuclei are a product of early events especially in oral regions because they are virtually absent in unexposed mucosa.

Correspondence: ***Dr. Apeksha Mainali****; e-mail:* ***drmainaliapeksha@gmail.com***

Classically, a micronucleus is defined as a small extranuclear chromatinic body originating from an accentric fragment or whole chromosome lost from the metaphase plate. Therefore, micronuclei frequencies have been considered to be reliable indices of both chromosome breakage and chromosome loss.[4] Micronuclei appear to be simple markers examined on routine cytopathological preparations. Their frequency of occurrence is a measure of chromosome breakage in early cell divisions and the number of micronuclei is known to increase with carcinogenic stimuli, long before the development of clinical symptoms. The use of fluorescence in the screening of micronucleus enhances the demonstration of nuclei and micronuclei.[4] A study was planned to assess the genotoxicity of varieties of arecanut and *Gutka* by evaluating the number of micronuclei in smears obtained from the buccal mucosa of arecanut (betel leaf, slaked lime) chewers and *Gutka* chewers. The aim of the study was to assess the genotoxic effects of arecanut and *Gutka*; and the objectives were to quantify the number of micronuclei in buccal mucosa of arecanut chewers; and to quantify the number of micronuclei in buccal mucosa of *Gutka* chewers.

MATERIALS AND METHODS

The study was conducted in Department of Oral Medicine and Radiology, Department of Oral Pathology and Microbiology, Manipal College of Dental Sciences, Mangalore and Department of Microbiology, Kasturba Medical College, Mangalore in the year 2007-2010.The study consisted of 140 individuals which included 3 groups. Group I was the control group that included 70 healthy individuals without any oral lesions or any habits of arecanut/*Gutka* chewing, alcohol consumption, smoking, tobacco chewing etc. Group II (subject) were arecanut chewers and Group III (subject) were *Gutka* chewers, with 35 individuals in each group. The examined population belonged to both urban and rural areas (Mangalore and surrounding places) with apparent good health and no immediate medical history. In the present study, the micronucleus test was applied to all 140 individuals.

Ethical clearance was taken from institutional ethical committee (KMC ethics Committee, Mangalore, Karnataka). Written consent were taken from the patients and interview based questionnaire about their habit were filled. The buccal mucosal scrapings from the controls and the subjects were smeared onto two grease-free glass slides for each case and immediately fixed in cytology fixative (50% ethyl alcohol and 50% ether). The staining procedure involved immersion of smeared, fixed slides in Acridine orange working solution and cells were counted under fluorescence microscope.

Cells were considered to contain micronuclei if the extranuclear content resembled the nuclei of the cell but was smaller in size, round to ovoid in shape with distinct outlines, non-refractile with the same colour as that of the main nucleus and which had a diameter less than 1/3rd of the main nucleus. A total of 500 cells were counted in the smears from the affected and unaffected site of each patient, thus 1000 cells per individual were counted.

The stained slides were interpreted by single oral microbiologist. The results were tabulated and subjected to statistical analysis.

Micronucleated cells in the subjects of Group II (Arecanut chewers) and Group III (*Gutka* chewers) as well as cells without micronuclei in the subjects of Group I were photographed.

Frequency tables of the data collected were generated by the use of the stastical package SPSS 11.5. Descriptive analysis was done and tests of association were carried out using $\chi 2$ (Chi-square test) statistics. The data followed a gaussian/normal distribution. Hence, a parametric test, i.e. Student's unpaired *t*-test was used to compare the mean values of different parameters of cases and controls.

RESULT

The practice of chewing arecanut products was found to be more prevalent among males compared to females with red variety of arecanut being more frequently consumed compared to white variety. The mean age of

Group I subjects was 34.22 years with the age range between 19-60 years. The mean age of Group II subjects was 44.51 years ranging between 28-70 years. The mean age of Group III subjects was 31.61 years ranging between 21-61 years.

Out of the two varieties of arecanut, 80% were red variety and the rest 20% were white variety of arecanut. The most commonly placed site in both arecanut and *Gutka* chewers was right buccal mucosa (57.1% and 51.4% respectively). The other sites commonly placed in decreasing order were both right and left sides and left buccal mucosa. 74.3% of the chewers spit and 25.7% swallowed arecanut and *Gutka* .

Various habit patterns of arecanut chewers and *Gutka* chewers in terms of duration (years of chewing habit), frequency (number of arecanut and *Gutka* consumed per day) and duration of placement of arecanut and *Gutka* (in minutes) in the oral cavity were also evaluated. The mean duration of chewing was 7.77 min and 6.91mm in arecanut chewers and *Gutka* chewers respectively (Table 1). The mean frequency of chewing per day was 3.54 min and 4.22 min in arecanut chewers and *Gutka* chewers respectively. The mean duration of placement in mouth was 3.94 minutes and 3.74 minutes in arecanut chewers and *Gutka* chewers respectively. Student's unpaired t test was done (t=0.853, 0.890, 0.859) and p value was found to be non significant in both the groups in terms of habit patterns (p> 0.05).

Table no.1: Demographic data and duration of chewing habit

Habit Pattern	Gender (Total no.70)	Mean Age Group	Mean Years of Chewing habit	Mean Frequency of chewing per day	Mean Duration of Chewing(in minutes)
Arecanut chewers	Male-6 Female-29	44.51	9.6	3.4	3.94
Gutka Chewers	Male-35 Female-0	31.61	14	4.22	3.74

Table 2: Comparison between the frequencies of occurrence of Micronuclei in the Right Buccal Mucosa of Chewers (Arecanut/*Gutka*) and Controls (non-chewing, healthy individuals)

Occurrence of Micronuclei	Number of Individuals (%)		Total (%)
	Controls	Chewers	
None	5 (41.7)	4 (5.7)	9 (11.0)
1.00	7 (58.3)	26 (37.1)*	33 (40.2)
2.00	0	25 (35.7)*	25 (30.5)
3.00	0	12 (17.1)*	12 (14.6)
4.00	0	3 (4.3)*	3 (3.7)
Total (%)	12 (100)	70 (100)*	82 (100)

* Statistically significant; *p < 0.001* when compared with the Control (non-chewing) group

Table 3: Comparison between the frequencies of occurrence of Micronuclei in the Left Buccal Mucosa of Chewers (Arecanut/*Gutka*) and Controls (non-chewing, healthy individuals)

Occurrence of Micronuclei	Number of Individuals (%)		Total (%)
	Controls	Chewers	
None	7 (58.3)	14 (20.0)	21 (25.6)
1.00	5 (41.7)	42 (60.0)*	47 (57.3)
2.00	0	11 (15.7)*	11 (13.4)
3.00	0	2 (2.9)*	2 (2.4)
4.00	0	1 (1.4)*	1 (1.2)
Total (%)	12 (100)	70 (100)*	82 (100)

* Statistically significant; *p < 0.001* when compared with the Control (non-chewing) group

Table 4: Comparison between the occurrence of Micronuclei in both sides of the Buccal Mucosa in Controls (non-chewers) and Chewers (Arecanut/*Gutka*)

Occurrence of Micronuclei	Number of Individuals (%)		Total (%)
	Controls	Chewers	
1.00	12	18 (25.7)	30 (36.6)
2.00	0	11 (15.7)*	11 (13.4)
3.00	0	23 (32.9)*	23 (28.0)
4.00	0	9 (12.9)*	9 (11.0)
5.00	0	5 (7.1)*	5 (6.1)
6.00	0	2 (2.9)*	2 (2.4)
7.00	0	2 (2.9)*	2 (2.4)
Total	12 (100)	70 (100)*	82 (100)

* Statistically significant; *p < 0.001* when compared with the Control (non-chewing) group

Table 5: Comparison of the occurrence of Micronuclei in the groups – Arecanut chewers, *Gutka* chewers and Controls (Healthy, non-chewing individuals)

Occurrence of Micronuclei	Number of Individuals (%)			Total (%)
	Arecanut chewers	*Gutka* Chewers	Controls	
1.00	9 (25.7)	9 (25.7)	12 (100)	30 (36.6)
2.00	7 (20.0)*	4 (11.4)*	0	11 (13.4)
3.00	9 (25.7)*	14 (40.0)*	0	23 (28.0)
4.00	5 (14.3)*	4 (11.4)*	0	9 (11.0)
5.00	3 (8.6)*	2 (5.7)*	0	5 (6.1)
6.00	1 (2.9)*	1 (2.9)*	0	2 (2.4)
7.00	1 (2.9)*	1 (2.9)*	0	2 (2.4)
Total (%)	35 (100)	35 (100)	12 (100)	82 (100)

* Statistically significant; *P = 0.008*, when compared to the Control (Non-chewing) group

The number of micronuclei in buccal mucosa of arecanut chewers and *Gutka* chewers were similar. Statistical analysis using the Chi-square test ($\chi 2$= 24.975; p=.002) showed that there was high significant difference in the frequency of micronuclei right buccal mucosa between arecanut chewers, *Gutka* chewers and individuals with no habit(Fig. no.1,Table 2).The Comparison of frequency of micronuclei in left buccal mucosa between arecanut chewers, *Gutka* chewers and individuals with no habit showed $\chi 2$ value of 13.791 and p=.087 stating that the results were non significant (Table 3) .

A comparison of the total number of micronuclei occurring in both right and left buccal mucosa between the controls and chewers was done which showed p value (p< .001) stating that the results were very highly significant. Cell counts per 500 intact cells each from right and left buccal mucosa indicated that the number of micronucleated cells was 198 from the chewers when compared with 12 from the controls (Table no.4).

The Comparison of total number of micronuclei in the buccal mucosa between arecanut chewers, *Gutka* chewers and individuals with no habit showed $\chi 2$ value of 26.962 and p=.008 stating that the results were highly significant. The mean percentage occurrence of micronucleated cells in the chewers was found to be 0.28%. (Table no.5)

DISCUSSION

Arecanut is an ancient, socially acceptable habit in South-East Asia. The usage of arecanut is indigenous to India, Srilanka, Maldives, Bangladesh, Myanmar, Taiwan and numerous islands in South Pacific; and also popular in parts of Nepal, Thailand, Indonesia, Malaysia, Cambodia, Vietnam, Philippines, Laos, China and in migrant communities from these countries. In populations resident in South and

East Asia, the use of arecanut is strongly interwoven into local art and craft, social customs, religious practices and cultural rituals. Betel quid chewing also is an ancient, socially acceptable habit especially in India.[5] Betel quid chewing also is an ancient, socially acceptable habit especially in India.[5] A major change in arecanut use occurred in India when an industrially manufactured mixture of arecanut, lime, catechin containing substance, sandalwood fragrance was introduced to the market in small aluminium foil sachets. This product was termed as *Gutka*. In a survey of 1200 students from colleges of Maharashtra, 9.6% chewed *Gutka*.[6] A study conducted in 1998 among 400 medical students in Patna, Bihar, India (out of a total of 509) revealed that about 12.5% were regular users of *Gutka* and the rest used other tobacco products not containing arecanut.[7]

In the present study, 55.7% of the chewers were males whereas 44% of the chewers were females. The higher popularity of chewing arecanut and *Gutka* amongst males was quite similar to those reported by earlier studies, such as 9.8%, 25.5%.[8,9] Our study result regarding chewing arecanut products being more prevalent among males compared to females is consistent with previously reported studies. Although a quantitative relationship between the number of quid consumed per day and the risk of developing genotoxic damage has been reported (Wahi, 1968)[10], we did not find any correlation between duration, frequency of habits, age, site of placement, and the frequency of micronuclei in the 2 habit groups. In a study by Nair et al (1991)[11] there was no correlation between the frequency and duration of chewing (tobacco and betel quid) habits and the frequency of micronucleated oral mucosal cells. A similar observation was also made in our study. This could be attributed to the short turn-over period of 25 days for oral mucosal cells.[12] Hence, the inference can be made that the cytogenetic damage in oral cells is acute and local. A normal cell undergoing mitosis in the presence of a carcinogen may become a micronucleated cell, due to a segregational defect during telophase or due to chromosomal breakage.[13] The results of this study showed that there was a significant elevation in micronucleated cells from the exfoliated oral mucosal cells obtained from arecanut chewers and *Gutka* chewers over control samples. However, no significant difference was observed between the two habit groups, arecanut and *Gutka*. This was consistent with the result obtained by Nair U (1991)[11] who measured the frequency of micronucleated cells (MNC) derived from exfoliated human oral mucosal cells to assess genotoxic damage in chewers of betel quid with tobacco and tobacco with lime. Significantly elevated frequencies of MNC were observed in the exposed groups (BQT = 4.83 +/- 0.70; T = 5.20 +/- 0.66 per 1000 cells) compared to the control group (C = 2.59 +/- 0.37). The percentage occurrence of micronucleated cells was 0.28% in arecanut and *Gutka* chewers. Stich and Rosin (1984)[14] mentioned the percentage micronucleated cells in tobacco chewers in the range of 2.18% to 7.25%. Regional variation is seen in the frequency in normal population as shown by the different values in different studies. Also, in our study, it was seen that the practice of chewing Gutka was popular among youngsters .Stich and Rosin (1984)[14] quoted the mean percentage occurrence of micronucleated cells in the control populations of different countries as 0.44% ranging from 0.0% to 0.9%.

CONCLUSION

Numerous comparisons carried out between arecanut, *Gutka* chewers and individuals with no habit revealed increase in the number of micronucleated cells in chewers as compared to individuals with no habit which reinforced the possible genotoxic damage leading to oral cancer in chewers and therefore the use of such practice should be strongly discouraged.

Acknowledgement: I would like to thank all the people involved directly/indirectly in the study.

Footnote: The paper was presented as an invited poster at the Supportive Care in Cancer MASCC/ISOO 2015 International Symposium in Copenhagen,Denmark on June 25-27, 2015.

REFERENCES

1. *Canniff J.P, Harvey W, Harris M. Oral submucous fibrosis: its pathogenesis and management. Br Dent J 1986; 160: 429-34.*
2. *Sinor PN, Gupta PC, Murti PR, Bhonsle RB, Daftary DK, Mehta FS, Pindborg JJ. A case-control study of oral submucous fibrosis with special reference to etiologic role of arecanut. Oral Pathol Med 1990; 19: 94-8.*
3. *Setty S, Ganapathy KS, Devi G, C Ramesh. Application of the micronucleus test to exfoliated epithelial cells from the oral cavity of beedi smokers, a high-risk group for oral cancer. Mutat Res 2004; 561(1-2):15-21.*
4. *Dias VM, Manelli-Oliveira R, Machado-Santelli GM. Using fluorescence for improvement of the quantitative analysis of micronucleus in cell culture. Mutat Res 2005; 565(2):173-9.*
5. *Strickland SS. Anthropological perspectives on use of the arecanut. Addiction Biology 2002; 7: 85-97.*
6. *Gupta PC, Ray CS. Tobacco and Youth in the South East Asian region. Ind J Cancer 2002; 39:5-35.*
7. *Hans G. Prevention of cancer in youth with particular reference to intake of pan masala and gutka. Mumbai, India: NSS Unit, TISS 1998.*
8. *Gupta PC. Epidemiology of Betel Quid Usage. Ann Acad Med Singapore 2004; 33 (Suppl): 31S-36.*
9. *Gunaseelan R, Sankaralingam S. Arecanut use in rural Tamil Nadu: A growing threat. Indian Journal of Medical Sciences, Jun 2007.*
10. *Wahi PN. The epidemiology of oral and pharyngeal cancer, Bull. W.H.O., 38, 495-521.*
11. *Nair U, Obe G, Nair J, Maru GB, Bhide SV, Pieper R, Bartsch H. 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 Nov; 261(3):163-8.*
12. *Squier CA, Finkelstein MW. Oral Mucosa in Oral Histology, Development, Structure & Function, 5th ed., Mosby Publications, pp. 351–2.*
13. *Heddle JA, Carrano AV. The DNA content of micronuclei in mouse bone marrow by gamma radiation- evidence that micronuclei arise from acentric fragment. Mutat Res 49 (1977) 63–9.*
14. *Stich H.F, Rosin M.P. Micronuclei in exfoliated human cells as a tool for studies in cancer risk and cancer intervention. Cancer Lett 1984; 22: 241–53.*