eISSN: 09748369, www.biolmedonline.com

Micronucleus investigation in buccal mucosal cells among pan masala/gutkha chewers and its relevance for oral cancer

Fareed M, Afzal M, *Siddique YH

Section of Genetics, Department of Zoology, Faculty of Life Sciences, Aligarh Muslim University, Aligarh 202002, U.P., India.

***Corresponding Author:** yasir_hasansiddique@rediffmail.com

Abstract

Micronuclei (MN) have been proposed as good biomarker to assess cytogenetic damage in biomonitoring studies. The analysis of MN in the epithelial cells has been shown to be a sensitive method for monitoring genetic damage in human populations. MN formation has been observed in pre-cancerous lesions of the oral cavity of pan masala and gutkha chewers. Increased cytogenetic damage has been observed in peripheral blood lymphocytes and exfoliated buccal mucosal cells of pan masala chewers. MN frequency obtained in case of pan masala/ gutkha chewers is very high as compared to control, which clearly indicates that chewers are at high risk for developing oral cancer.

Keywords: Micronucleus (MN); pan masala; gutkha; buccal mucosal cells; oral cancer.

Introduction

Cytogenetic markers like chromosomal aberrations (CAs), sister chromatid exchanges (SCEs), and micronuclei (MN) are sensitive indicators of genetic damage. Micronuclei (MN) are small chromatin bodies that appear in the cytoplasm by the condensation of acrocentric chromosomal fragments or by whole chromosomes, lagging behind the cell division. Thus, it is the only biomarker that allows the simultaneous evaluation of both clastogenic and aneugenic effects in a wide range of cells, which are easily detected in interphase cells. Compared with healthy controls, all the habit groups, irrespective of their type of chewing, had significantly higher frequencies of micronucleated exfoliated oral mucosal cells (Kayal et al., 1993). The main carcinogens in pan masala and gutkha are derived from their ingredients; areca nut, catechu, lime and tobacco. Reactive oxygen species (ROS), implicated in multistage carcinogenesis, are generated in substantial amounts in the oral cavity during chewing (Nair et al., 1992). Gutkha and pan masala are dry products and one can assume that the ROS concentration will increase in the oral cavity of chewers as soon as the areca nut and catechu polyphenols together with slaked lime dissolve in the saliva, similar to the reaction observed in vitro (Nair et al., 1987). High copper and iron content in these products would further add to the load through their action as catalysts via the Haber-Weiss and Fenton reactions. This has been demonstrated in primary human oral keratinocytes, where pan masala induces superoxide radical production and lipid peroxidation. The mutagenic, clastogenic and carcinogenic properties of areca nut, the major constituent of pan masala, have been extensively studied in a variety of experimental systems (Jeng et al., 2001). Areca nut chewing has been classified as carcinogenic to humans (IARC, 2004). Areca nut contains 5-40% polyphenols and several alkaloids including arecoline, arecaidine, guvacine and guvacoline. Arecoline, the most important areca nut alkaloid, is present at 1% of the dry weight and has been shown to be genotoxic (Dave et al., 1992a). Pan masala applied to the palate and cheek mucosa of albino Wistar rats resulted in keratosis, thickening of the submucosal collagen, an inflammatory reaction and changes in tissue vasculature, similar to those observed in oral submucous fibrosis and leukoplakia in humans (Khrime et al., 1991). Tissue inhibitors of metalloproteinases (TIMPs) and matrix metalloproteinases (MMPs) are the major gelatinolytic proteinases secreted by human mucosal fibroblasts. Arecoline treatment alters the balance in favour of matrix stability by elevating TIMP-1 expression and inhibiting MMP-2 activity, which could lead to development of fibrosis in chewers (Chang et al., 2002). The frequencies of sister chromatid exchanges and chromosome aberrations in peripheral blood lymphocytes and the percentage of micronucleated exfoliated cells of buccal mucosa were significantly increased among areca nut chewing, oral submucous fibrosis (OSF) and oral cancer patients compared with those of non-chewing controls (Dave et al., 1992b). DNA aneuploidy in oral

leukoplakia in Caucasian tobacco users have been found to signal a very high risk for subsequent development of oral squamous cell carcinomas and associated mortality (Sudbo and Reith, 2003; Sudbo et al., 2004). Both tobacco and areca nut are also major risk factors for oral leukoplakia (Trivedy et al., 2002). Oral leukoplakia is well recognized for its potential for developing into oral cancer. Transformation rates for malignant change have been reported from 0.1 to 17.5% (van der Waal et al., 1997). A dose–response relationship has been suggested by an increasing relative risk with increasing frequency of areca nut chewing (Sinor et al., 1990; Hazare et al., 1998; Lee et al., 2003).

Materials and Methods

(a) Chemicals
Trizma hydrochloride ethylene diamine tetraacetic acid (EDTA) from SRL, India. Giemsa stain, sodium chloride, methanol, glycerol and sodium hydroxide pellets from Merck (India).

(b) Oral mucosa cell collection
Oral mucosa cells were collected from each subject using a soft toothbrush gently from the oral mucosa of cheeks (Surralles et al., 1997). The brush was then swirled into a centrifuge tube containing a buffer solution of pH 7.0 thereby creating a cell suspension.

(c) Preparation of 0.001 Tris HCl, pH 7.0 buffer
To make the buffer solution (0.1M EDTA, 0.001M Tris HCl and 0.02M NaCl) were dissolved in sterile 1 litre distilled water. The pH of the buffer was adjusted to 7.0 with NaOH.

(d) MN assay in oral mucosal cells
Oral mucosa cells were washed thrice by centrifugation at 1500 rpm for 10 minutes in the buffer solution (Surralles et al.,1997). Volumes of 25 ml of buffer in a 50 ml conical tube was used in every washing step. Washing with the buffer leads to the inactivation of endogenous DNAases present in the oral cavity, remove bacteria and cell debris that would complicate the scoring (Titenko-Holland et al., 1994). Gentle pipetting of the cells in the buffer solution reduces clumping and lyses broken cells. The cell density was checked with a phase contrast microscope. The cell solution was either concentrated by centrifugation or diluted in the buffer as required. Once the cell density (1.5-2 x 10^6/ml) was reached, 50-100 µl of the cell suspension was laid and spread well on clean, pre-heated (37^oC) glass slide and allowed to air dry for 5-10 minutes. The slides were fixed in methanol, stained with 5% Giemsa (Rajeshwari et al., 2000) and observed under microscope. About 1500 oral mucosal cells were scored per individual.

(e) Scoring procedure and criteria
All the MN in oral mucosa cells were scored in accordance with the criteria reported by Tolbert et al., 1992. All slides were first examined with low power magnification to discard those infected with bacteria and fungi as they interfere with scoring. Only those MN were scored which were rounded or oval in shape with a smooth perimeter suggestive of membrane, less than $^1/_3$ the diameter of the main nucleus, of the same, texture and color and refraction as the main nucleus and clearly separated from the main nucleus with no overlap or bridge to nucleus. Criteria for the cells to be included in the total count were: intact cytoplasm lying relatively flat, little or no overlap with adjacent cells, little or no debris, nucleus normal and intact, and nuclear perimeter smooth and distinct. Two trained persons crosschecked all MN scores to obviate the risk of bias. All questionable MN were additionally assessed by a third scorer and discussed until a consensus were reached.

Results
The MN frequencies obtained during the present investigation are presented in Tables 1-3, which show MN frequency to the greater in exposed cases (3.56±0.719) as compared to the controls (0.75±0.171).

Table 1: Micronucleus distribution in normal individuals (control).

S. No.	Micronucleus				Micronucleated cells	Total Micronucleus
	1	2	3	4		
1	1	-	-	-	1	1
2	-	1	-	-	1	2
3	1	-	-	-	1	1
4	-	-	-	-	0	0
5	-	-	-	-	0	0
6	1	-	-	-	1	1
7	-	-	-	-	0	0
8	-	-	-	-	0	0
9	1	-	-	-	1	1
10	1	-	-	-	1	1
11	-	-	-	-	0	0
12	1	-	-	-	1	1
13	2	-	-	-	2	2
14	-	-	-	-	0	0
15	1	-	-	-	1	1
16	1	-	-	-	1	1
Total	10	1	0	0	11	12

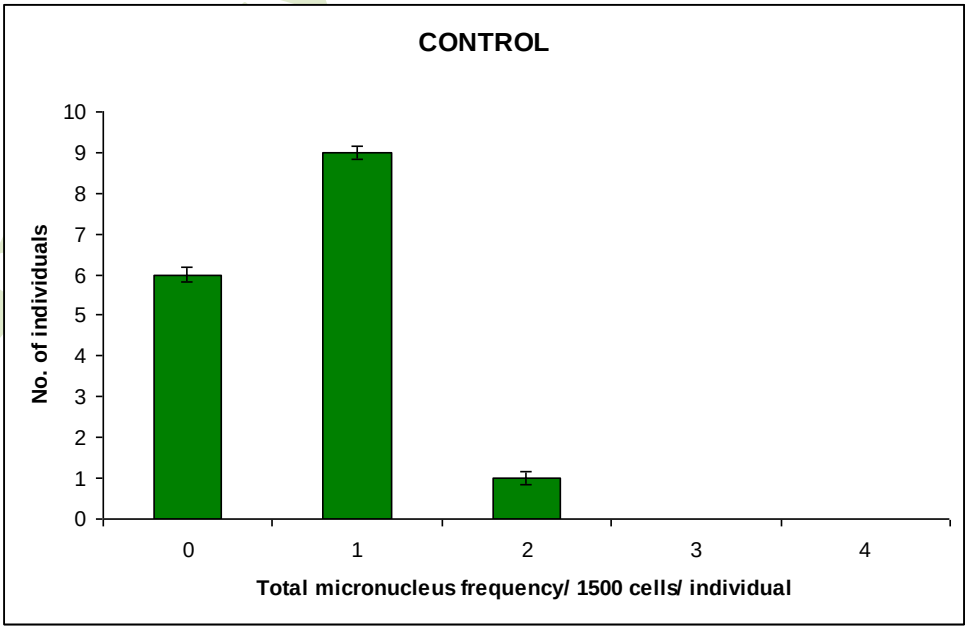
Table 2: Micronucleus distribution in individuals using pan masala/gutkha (cases).

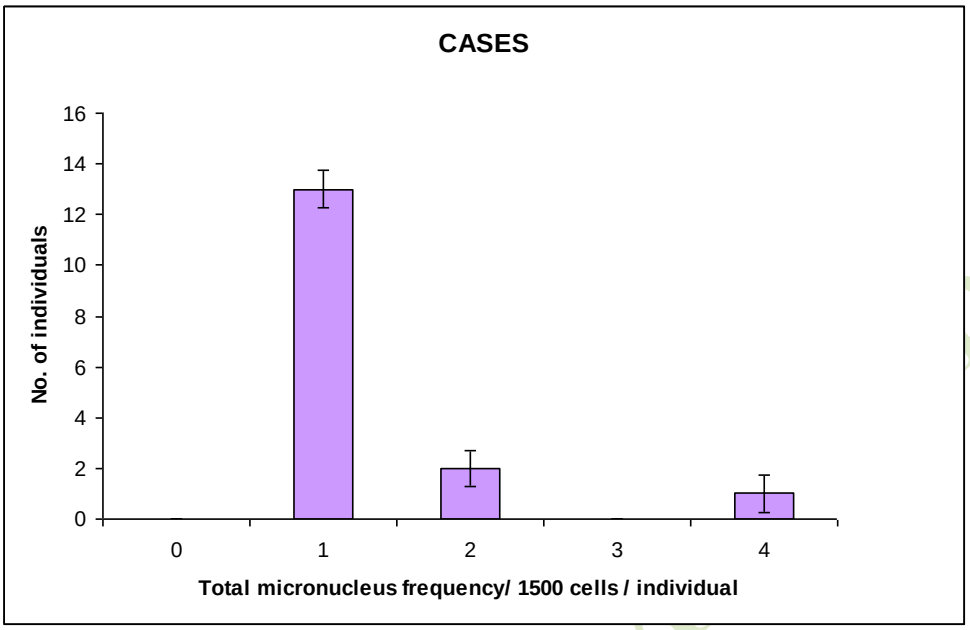
S. No.	Micronucleus				Micronucleated cells	Total Micronucleus
	1	2	3	4		
1	7	2	-	-	9	11
2	4	-	-	-	4	4
3	3	-	-	-	3	3
4	2	-	-	-	2	2
5	3	-	-	-	3	3
6	4	2	-	-	6	8
7	4	-	-	1	5	8
8	2	-	-	-	2	2
9	2	-	-	-	2	2
10	1	-	-	-	1	1
11	3	-	-	-	3	3
12	1	-	-	-	1	1
13	2	-	-	-	2	2
14	2	-	-	-	2	2
15	2	-	-	-	2	2
16	3	-	-	-	3	3
Total	45	4	0	1	50	57

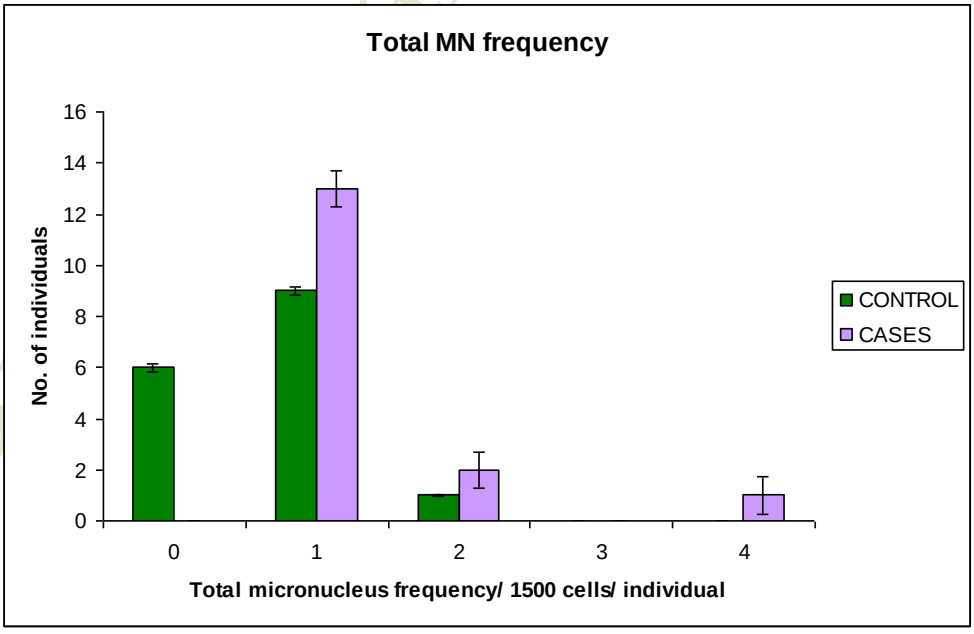
Table 3: Total micronucleus frequency per 1500 cells per individual in buccal region of 16 cases and 16 controls, with their respective means and standard errors.

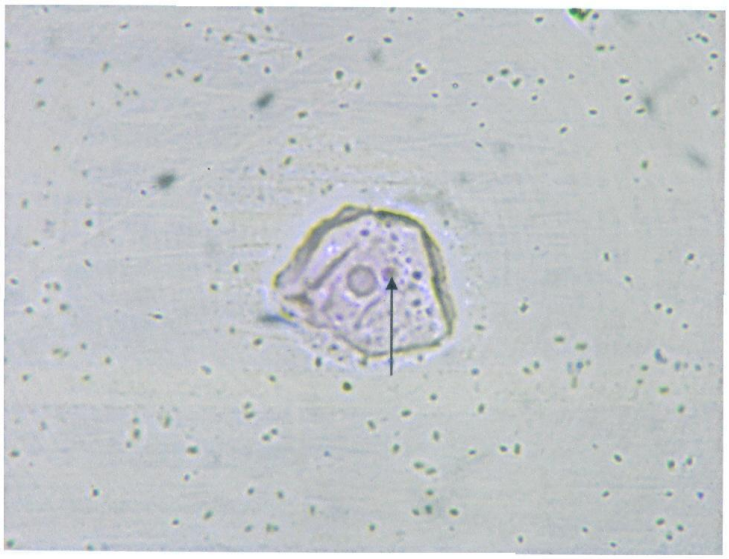
Individual	Cases		Control	
	Age (years)	TMN	Age (years)	TMN
1	36	11	41	1
2	30	4	34	2
3	38	3	25	1
4	55	2	22	0
5	25	3	22	0
6	42	8	22	1
7	40	8	32	0
8	31	2	24	0
9	30	2	24	1
10	33	1	23	1
11	45	3	22	0
12	19	1	22	1
13	28	2	22	2
14	35	2	23	0
15	20	2	22	1
16	40	3	23	1
Mean ± S.E.	3.56 ± 0.719*		0.75 ± 0.171*	

*p<0.01 (Significant with respect to control)




CASES
No. of individuals
16
14
12
10
8
6
4
2
0
0
1
2
3
4
Total micronucleus frequency/ 1500 cells / individual


Total MN frequency
No. of individuals
16
14
12
10
8
6
4
2
0
0
1
2
3
4
Total micronucleus frequency/ 1500 cells/ individual
CONTROL
CASES



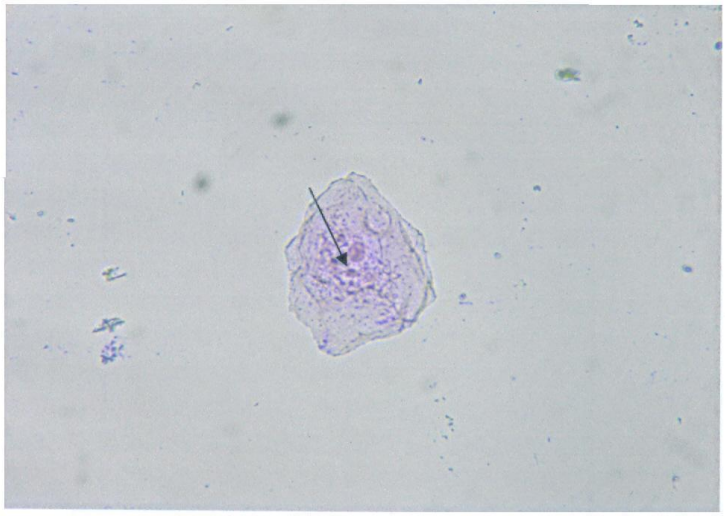


Plate 1: Oral buccal mucosal cells having micronucleus.

Discussion

Micronucleus (MN) has been used since 1937 as an indicator of genotoxic exposition based on radiation studies conducted by Brenneke and Mather, as reported by Heddle et al., (1983). Since the prolonged use of the chewing items such as supari, pan masala/gutkha can generate a risk of developing different types of oral cancer, it becomes necessary to screen the population for its possible risk. Gutkha and pan masala have been shown to be carcinogenic in experimental animals, causing tumors in various organs. Pan masala acts as a tumor promoter in mice (Ramchandani et al., 1998). Mice fed pan masala developed tumors of the lung, liver, stomach and testis (Bhisey et al., 1999). Pan masala reduced testis weight in mice and enhanced the frequency of morphological abnormalities in mouse sperm (Kumar et al., 2003). Swiss mice fed gutkha or pan masala in the diet developed tumors affecting various organs such as lung, stomach, liver, testis, ovary and adrenal gland, which showed that gutkha being more potent than pan masala (Nigam et al., 2001).

From the present study, the gradient of MN frequencies in oral buccal region of cases (3.56 ± 0.719) is significant in comparison to control (0.75 ± 0.171). Positive associations between oral cancer and the habit of chewing areca quid have been reported (Das and Dash, 1992; Gupta, P.C., 1991; Reichart et al., 1995). Similarly, increase in frequencies of MN in pan masala and gutkha consumers have also been reported (Siddique et al., 2008; Gandhi and Kour 2000). MN formation has been observed in precancerous lesions of the oral cavity of chewers (Nair et al., 1991). In our study, mild lesions and ulcers with pain are also observed in the buccal region of gutkha and pan masala habituals between the age of 30-50. Similar age-standardized result was reported by Gupta P.C., (1999).

Conclusion

From the present study, increase in MN frequency provides the evidence that gutkha and pan masala chewers may be at high risk for developing oral cancer. Hence, the MN assay can be used as a biomarker of genotoxicity, and the further study of large groups of cases and controls will strengthen the findings of the present study.

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