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CYTOGENETIC ANALYSIS OF MICRONUCLEI, SISTER CHROMATID EXCHANGE AND CHROMOSOMAL ABERRATIONS IN PAN MASALA CHEWERS

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ABSTRACT

Pan Masala (PM) chewing is very dangerous for health but it is becoming very popular day by day. PM is a dried powdered mixture containing ingredients like areca nut, catechu, lime, cardamom and flavouring agents. It is consumed abundantly by Indians and is also exported to Western countries. A cytogenetic study to assess the micronucleus (MN), sister chromatid exchange (SCE) levels and chromosomal aberrations among (CA) 60 pan chewers and 60 non-chewers was conducted in Chennai, Tamil Nadu. In the present cytogenetic monitoring study, analysis of MN was significantly higher (15.82 ± 1.31) in chewers than controls (4.82 ± 1.47) ($P < 0.001$) and SCE also was significantly higher in chewers (9.23± 2.12) than controls (4.80±1.11) ($P < 0.001$). In exfoliated buccal mucosa and chromosome analysis (CA), frequency of chromatid type aberrations is lower in controls than chewers such as gaps (0.90% v. 1.83%) breaks (0.47% v. 1.77%), exchanges (0.02% v. 0.18) and acentric fragments (0.20% v. 0.90%). The increased percentage of aberrations found among pan chewers is significantly higher when compared to that of the controls. Isochromatid aberrations also increased significantly such as gaps (0.12% v. 0.97%) breaks (0.07% v. 0.80%), acentric fragments (0.05% v. 0.23%), dicentrics (0.02% v. 0.63%), and these were estimated in the peripheral blood lymphocytes to assess possible DNA damage. All three cytogenetic endpoints demonstrated a statistically significant increase ($P < 0.001$) among the pan masala chewers as compared with the non-consuming controls. In conclusion, we propose the use of the cytokinesis-block micronucleus assay, sister chromatid exchange and chromosome aberrations for getting a sensitive information on cell cycle progression and cytotoxicity of chewing pan masala.

KEYWORDS

Micronuclei, Chromosome aberration, Sister chromatid exchange, Pan masala, Buccal mucosa.

INTRODUCTION

There is an increasing effort world-wide to determine the impact of environmental, genetic and life- style factors on genomic stability in human populations. As a result of rapid globalization and changing social attitudes, tobacco and betel quid chewing habits have been increasing worldwide. Tobacco chewing along with various ingredients like areca nut, catechu, lime, cardamom, permitted spices, unspecified flavouring agents have been reported to possess cytotoxic, mutagenic and genotoxic properties (1). Betel quid chewing is an ancient practice common in many Asian countries. Betel quid generally consists of betel leaf, areca nut, and slaked lime, to which tobacco is often added (2). Regular chewing of betel quid has several adverse effects on the oral cavity and upper digestive tract, including inflammation, development of white or gray patches on the tongue and buccal mucosa, and oral cancer.

Micronuclei are small, extranuclear bodies that arise from acentric chromosome fragments or from whole chromosomes that are excluded from the nucleus during mitotic cellular division. They can be a consequence of DNA breakage, replication on a damaged DNA template or inhibition of DNA synthesis, failure of any of the mitotic apparatus or alterations in cellular physiology and mechanical disruption (3). Micronucleus analysis can be used for a number of cells, both in vitro and in vivo, including lymphocytes (4) and buccal epithelial cells (5). Micronucleus induction is an indirect indicator of mutagenicity, the frequency of micronuclei was significantly higher in the exposed group than in the controls and there was also a positive correlation between the number of years of exposure and frequency of micronuclei (6). The micronuclei test is the most frequent technique used to detect chromosome breakage or mitotic interference thought to be associated with increased risk for cancer (7).The frequency of micronucleated cell was measured to assess genotoxic damage in betel quid chewers. When compared to other body sites, the mouth offers a unique opportunity to define biomarkers because the mouth permits non-invasive examination in longitudinal studies of smoking and smokeless tobacco-associated acute and chronic diseases.

Structural chromosome aberrations arise from direct DNA breakage, replication on a damaged DNA template or inhibition of DNA synthesis and may involve both chromatids of the chromosome or only one chromatid of the chromosome (8). Chromosome aberration analysis has been commonly performed on human peripheral blood lymphocytes to assess DNA damage (9). To ensure that only first-generation metaphase cells are scored for CA, bromodeoxyuridine is commonly added to the culture medium prior to DNA replication in vitro (10). The induction of SCE has been widely used as an indicator of DNA damage following exposure to pesticides (11). A significant higher frequency of percentage micronucleated cells in exfoliated buccal mucosa; and increased frequency chromosomal aberrations (CA) and sister chromatid exchanges (SCE) in lymphocytes of chewers of Areca nut has been reported (12). Extract of pan masala increased the frequency of sister chromatid exchange and chromosomal aberrations in mouse bone marrow cells (13). Induction of SCE and dominant lethal mutation by 'Catechu' were studied on mice following acute and prolonged oral treatment (14). Epidemiological studies have shown an association between the habit of chewing betel- nut and risk of oral cancer in the Indian population (15).

Therefore in the present study, we aimed to analyze and compare micronuclei in exfoliated buccal mucosa, chromosome aberration and sister chromatid exchange from lymphocyte culture in chewers and in controls to identify, among these three biomarkers which is more reliable in terms of risk prediction for genotoxicity, estimate synergistic effect of tobacco exposure with level of biomarkers, and identify best cellular site of measurements for genotoxicity assessment.

MATERIALS AND METHODS

Study Sample

Blood samples were taken from120 healthy male individuals, 60 pan masala chewers and 60 control individuals, who had not been subjected to X-ray treatments or had used any medication during the last three months at the time of investigation. Samples were taken from the subjects living in and around Chennai and nearby villages. Most of the pan masala chewers screened in this study worked as gardeners, auto drivers, rickshaw pullers, factory labourers. Some of the subjects chewing pan masala belong to lower middle class families involved in building contract work. Care was taken to select individuals who have had the habit of chewing pan masala (without tobacco or other concurrent habit of tobacco) or had the habit of areca nut consumption only. The Control group comprised normal healthy individuals belonging to the same socio-economic group who did not have the habit of taking pan masala, areca nut or tobacco in any form, chewing or smoking. They hailed from the same places, viz., Chennai and the villages around, to which the exposed individuals belonged. Almost all the sampled individuals exposed as well as controls, took vegetarian diet (wheat, bread, dhal/vegetables) only. Both the groups kept away from alcohol consumption.

Collection of Exfoliated Buccal and Blood cells

Exfoliated buccal mucosa cells can be collected using a wooden tongue-depressor, a metal spatula, or a cytobrush moistened with water or buffer to swab or gently scrape the mucosa of the inner lining of one or both cheeks. The cells were smeared on clean glass slides, fixed with acetic acid: methanol (1:3) air-dried and were stored until staining. A few studies have used toothpicks or toothbrushes (16, 17). Blood samples were collected in disposables pre-sterlized heparinized syringes and transferred to laboratory without delay for lymphocyte culture. Short term lymphocyte cultures were set up using the technique of the previous method (18) with minor modifications used for CA and SCE analysis.

Culture of Buccal Mucosa Cells for Micronucleus Analysis

Exfoliated buccal mucosa cells were incubated at 37°C for 72 h in a 5% CO_2 atmosphere. The culture medium consisted of RPMI 1640 with 2Mm L-glutamin and hepes buffer (Gibco) supplemented with 15% fetal calf serum and antibiotics Phytohaemagglutinin (PHA M, Difco) at a concentration of 40 micro g / ml was used to stimulate cell division. Cytochelasin B (6 micro g /ml in DMSO, Sigma) was added 42 h after culture initiation in order to block cytokinesis. After an incubation period of 70 h, the cells were harvested, treated with a hypotonic solution of 0.075 M KCL, and fixed with a mixture of methanol-glacial acetic acid 3:1 following the protocol described by (19). Fixed cells were dropped onto clean microscopic slides, air dried and stained by the Giemsa technique. A standard set of scoring criteria, together with matching photomicrographs and line diagrams for the buccal MN assay, similar to those developed for the cytokinesis-block lymphocyte MN assay (20) were used in the research. To analyse the total number of MN in lymphocytes, a total of 1000 binucleated cells with well preserved cytoplasm were scored per subject on coded slides.

Culture of Peripheral Blood Lymphocytes for Chromosome Analysis

Heparinised blood samples were drawn from the subjects and controls and lymphocyte cultures were initiated with 0.5ml of whole blood in RPMI 1640 medium containing 20% AB serum, 0.5% phytohaemagglutinin and 0.25% antibiotic. The cultures were incubated at 37° C for 72 hours. Colchicine was added to the cultures at the 70th hour to arrest the cell cycle at metaphase stage. Cultures were harvested, slides were prepared, coded and air dried according to the standard method (18). Slides were stained using giemsa and each sample 100 well spread metaphases were screened for various structural and numerical aberrations.

Culture of Peripheral Blood Lymphocytes for Sister Chromatid Exchange Analysis

For sister chromatid exchanges, 5-bromodeoxyuridine (10 µg/ml, Sigma) was added 24 h after setting up the cultures. Cells were harvested after 72 h. Slides were prepared by air drying method and stained with Hoechst 33258 and 4% Giemsa, following the method (21). For calculating frequency of SCE per cell, 30 metaphases were analysed as per international practice. Micronucleus, sister chromatid exchange and well band metaphase chromosomes images were taken under the oil immersion objective in Olympus (BX 51) fluorescent microscope attached with COCHU high performance CCD camera with a magnification of 100X.

Statistical analysis

All data analyses were performed using the SPSS Version 17.0. Data were double entered, and the resulting data sets were compared and checked for completeness and accuracy of entry. The significance of differences between the mean for the two groups was assessed with Student's t-test, the differences in means were illustrated using box plots, relationships between micronuclei and chromatid exchange levels were assessed using correlation, and discriminant analysis has been used to differentiate the control and pan-chewers groups using micronuclei and chromatid levels. A p value < 0.05 was considered to be statistically significant. Descriptive statistics were applied for calculating the distribution of various characteristics.

RESULTS

The results obtained from micronuclei assay, sister chromatid exchange and chromosomal abnormalities are used to detect DNA damage among the pan chewers. A random sample of 60 people who never chewed any kind of pan and another group of the same sample size who have the habit of pan chewing is covered in the study. Three types of parameters such as MN, SCE and CA are measured among the two groups. The mean values and box plots of the MN and SCE values of the two groups are given in Table 1 and in Figure 1.

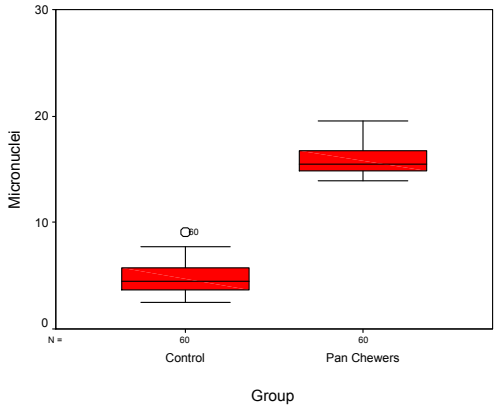
Table 1

Frequency of Micronuclei (MN) and Sister Chromatid Exchanges (SCE) in buccal mucosal and blood cells in pan masala chewers and controls

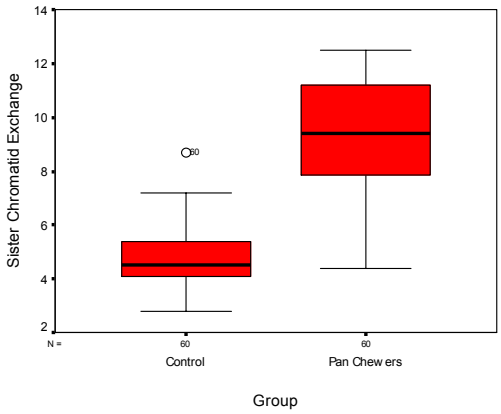
	Group	N	Mean	Std. Deviation	Std. Error Mean	t-test value	Sig.
Age	Control	60	35.07	9.363	1.209	-.777	.439
	Pan Chewers	60	36.33	8.483	1.095		
Micronuclei	Control	60	4.823	1.4726	.1901	-43.167	.000
	Pan Chewers	60	15.823	1.3144	.1697		
Sister chromatid exchange	Control	60	4.802	1.1176	.1443	-14.318	.000
	Pan Chewers	60	9.235	2.1221	.2740		

Values in percentage indicate damage cell per 1000 binucleated for MN and 30 metaphase for SCE.Values are significantly higher than the corresponding values for controls (t-test) p < 0. 001.

The average age of the two groups remains almost at the same level. Micronuclei and sister chromatid exchange values are found to be higher among pan chewers compared to the normal group. Independent samples t-test has been applied to verify whether the mean values of the micronuclei and sister chromatid exchange are the same between control and pan chewers groups.



Micronuclei



Sister chromatid exchange

Figure 1

Box plot analysis of Micronuclei and Sister Chromatid Exchanges for Control and Pan Chewers groups

It is found from the test that the mean values are not the same between pan chewers and control groups. Pan chewers have about four times higher micronuclei level compared to those who do not have the habit of pan chewing. As far as sister chromatid exchange is concerned, pan chewers has about two times higher level compared to non-pan chewers. The correlation analysis for the relationships between MN and SCE has been carried out for the two groups separately and the results are given in Table 2a. Correlations between duration of pan chewing and micronuclei and sister chromatid exchange levels for the pan chewers are given in Table 2b.

Table 2a

Correlations between Micronuclei and Sister Chromatid Exchanges for Control and Pan Chewers groups

Group		Micronuclei	Sister Chromatid Exchange
Control	Micronuclei	1	0.805(**)
	Sister chromatid exchange	0.805(**)	1
Pan Chewers	Micronuclei	1	0.186
	Sister chromatid exchange	0.186	1

*** Correlation is significant at the 0.01 level (2-tailed).*

Table 2b

Correlations between Micronuclei, Sister Chromatid Exchanges, and duration of Pan Chewing

	Micronuclei	Sister chromatid Exchange	Exposure duration in years
Micronuclei	1	0.186	0.588(**)
Sister Chromatid Exchange	0.186	1	0.261(*)
Exposure duration in years	0.588(**)	0.261(*)	1
** Correlation is significant at the 0.01 level (2-tailed).			
* Correlation is significant at the 0.05 level (2-tailed).			

The correlations between the duration of pan chewing and levels of MN and SCE are given in Table. The study result**s** showed that longer the duration of pan chewing, more will be the micronucleus and sister chromatid exchange levels as the correlations are found to be positive and statistically significant. Line diagrams of control and Pan chewing groups both for MN and SCN levels presented in Figure 2.

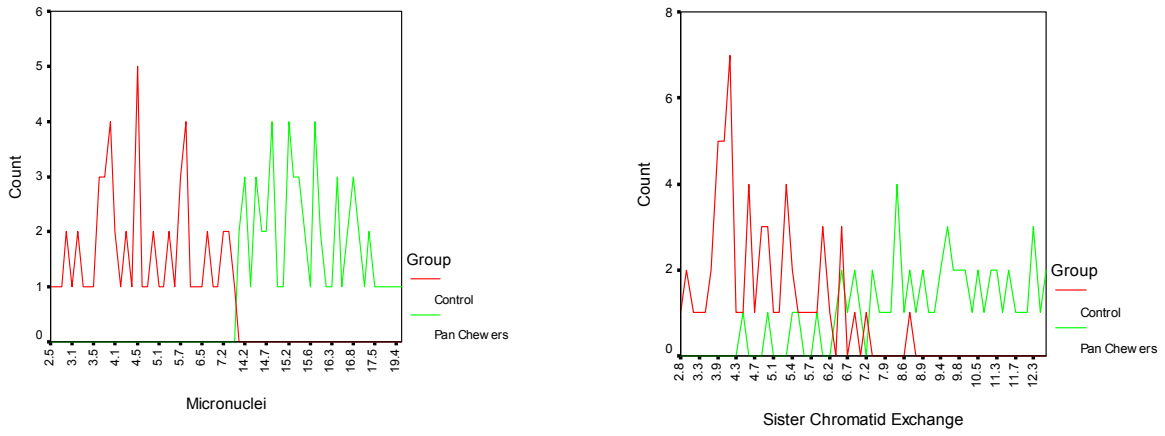


Figure 2

Line diagrams for Micronuclei and Sister Chromatid Exchange levels between Control and Pan Chewers groups.

Stepwise discriminant analysis clearly indicated that only the micronuclei level has the power to differentiate the two groups and sister chromatid exchange may not be used as a parameter to differentiate control and pan chewing groups. The discriminant function to differentiate the two groups is given in Table 3.

Table 3
Discriminant Function Coefficients – Unstandardized

	Function
	1
Micronuclei	0.716
(Constant)	-7.396

The discriminant function constructed by the analysis is given by: z = -7.396 + 0.716 x Micronuclei.

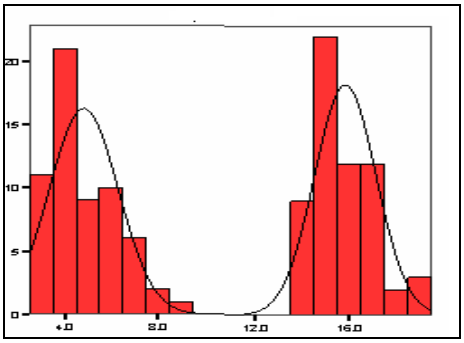
An individual is considered as belonging to control group if the z value is less than 0, or allocated to pan chewing group if the z value is greater than zero. Classification table is constructed to find out the efficiency of the functions constructed (Table 4). Normally the discriminant functions are said to be more effective for classification purposes, if the percentage of classification is more than 80%.

Table 4
Classification Statistics

			Predicted Group Membership		
	Group	Control	Pan Chewers		Total
Original	Count	Control	60	0	60
		Pan Chewers	0	60	60
	%	Control	100.0	.0	100.0
		Pan Chewers	.0	100.0	100.0

100.0% of original grouped cases correctly classified.

Since the classification is 100%, it may be concluded that the model z = -7.396 + 0.716 x Micronuclei is effective enough to identify a pan chewer from a normal person. The distribution of MN among the two groups is given in Figure 3.



Control Group Pan Chewers Group

Figure 3
Micronuclei Distribution – Control and Pan Chewers Groups

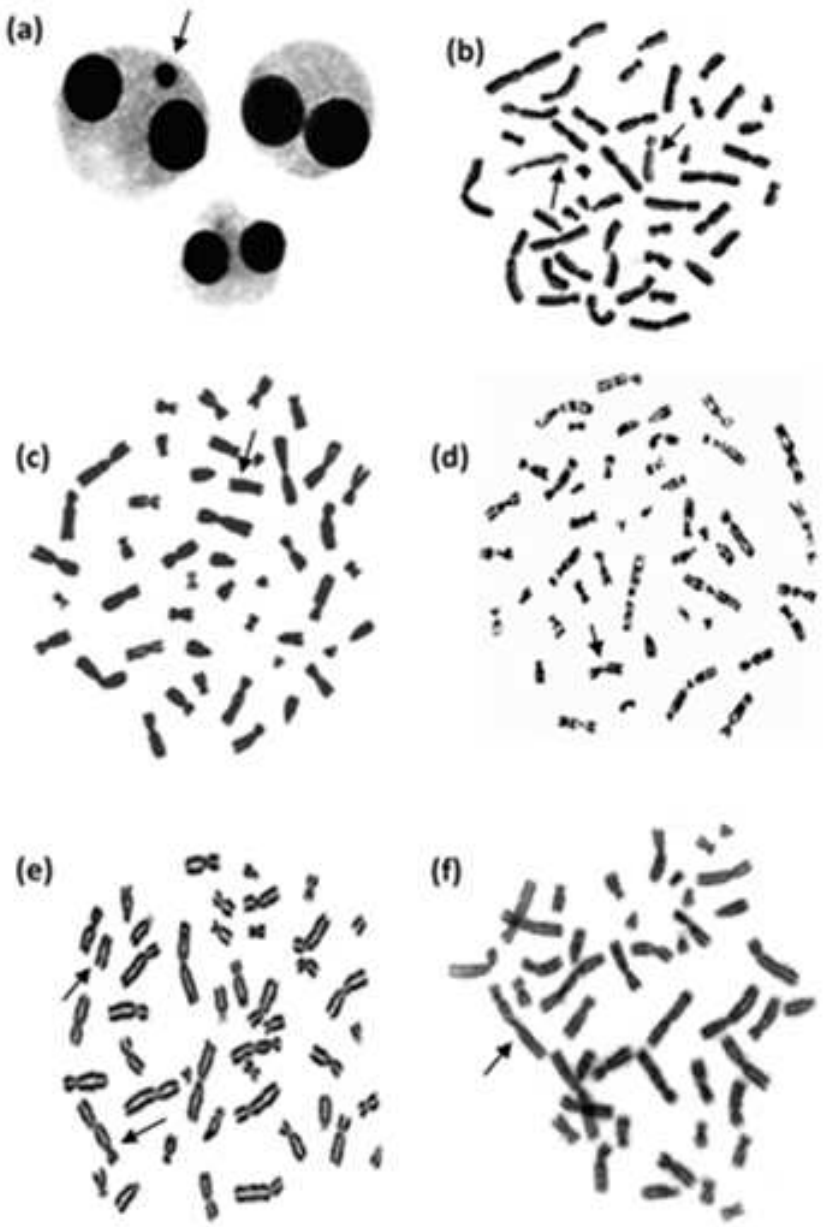
The incidence of chromosomal aberrations such as gaps, breaks, fragments and dicentrics in the pan chewers and matched controls was presented in Table 5 and Figure 4. The statistical proportions test has been applied for this study to test whether the proportions in each of chromosomal aberration types differ between the control and pan chewers groups, There was a significant increase in the frequency of total chromosomal aberrations in the pan chewers group when compare to the controls. The frequency of chromosomal aberrations like gaps, breaks, fragments and dicentrics which were recorded in the present study were both of chromatid and isochromatid aberrations.

Table 5
Frequency of Chromosomal Aberrations among Control and Pan Chewers

Details	Control Group	Control Group (%)	Pan Chewers Group	Pan Chewers Group (%)	Z - Values	Sig.
Chromatid type aberrations	54	.90	110	1.83	2.412	0.0160
Gaps						
Breaks	28	.47	106	1.77	3.711	0.0022
Exchange	1	.02	11	.18	1.582	0.1142
Acentric fragments	12	.20	54	.90	2.840	0.0046
Iso chromatid aberrations	7	.12	58	.97	3.474	0.0022
Gaps						
Breaks	4	.07	48	.80	3.349	0.0024
Acentric fragments	3	.05	14	.23	1.462	0.1442
Dicentric	1	.02	38	.63	3.250	0.0026

Values in percentage indicate Chromosomal aberrations per 100 metaphase. Values are significantly higher than the corresponding values for controls (Z-values) p < 0. 001.

Figure 4
Micronuclei and Chromosomal Aberrations in Pan Chewers



a. Micronuclei containing with binucleated cell ***b. Sister chromatid exchanges***
c. Acentric fragments ***d. Chromatid breaks and gaps***
e. Dicentrics and fragments ***f. Dicentrics and exchanges***

DISCUSSION

In the present study, we observed a low number of MN cells in the oral mucosa of the control groups (non-chewers). The results of pan masala consumers reported in this study have shown a significant increase of baseline micronuclei, sister chromatid exchange and chromosomal abnormalities. The micronucleus test has been receiving increased attention as a simple and sensitive short-term assay for detection of environmental genotoxicants (22). By applying this test, an elevated incidence of micronuclei has been recorded in the buccal mucosa cells of smokeless tobacco chewers. Sufficient and compelling evidence shows that the constituents of betel leaf, areca nut and tobacco have cytogenic, genotoxic and mutagenic effects on mammals. For example, these products enhance chromatid breakage and exchange in the range of 12-37% in human cells in vitro and DNA strand breakage in mouse kidney cells or human epithelial cells (23). The strong and intriguing relation between the use of betel quid and tobacco chewing was found to be a public health hazard. A similar work was conducted by (24) in North Indian subjects, and it was reported that the chewing of areca nut, alone or in combination with betel leaf and lime, caused damage to the oral mucosa.

A study done earlier (12) reported increased MN frequency in areca nut chewers than controls. In the present study, chewers were heterogeneous in terms of chewing mixture of tobacco, areca nut and other ingredients like catechu, lime and unspecified flavouring agents, MN was significantly higher (15.82 ± 1.31) in chewers than controls (4.82 ± 1.47) ($P < 0.001$). Such significant difference was also noticed in SCE (9.23 ± 2.12) among pan chewers than controls (4.80 ± 1.11) ($P < 0.001$). The study was observed that saliva of Pan bahar (a commercially available combination of ingredients like betel nut, catechu, lime, sandal oil, menthol, cardamom, flavor spices, fennel seeds, sugar, waxes, till seeds, colors, etc.) chewers was clastogenic to CHO cells (25). A very high frequency of MN has been observed among tobacco users (26, 27). Similarly increase in frequency of MN in “pan masala” consumers has also been reported by (28). The increase in the frequency of SCE has been reported in betel and tobacco chewers (29) . Sister chromatid exchange and chromosome aberrations were examined in peripheral blood lymphocytes and the frequency of micronucleated cells was scored in exfoliated buccal mucosa cells of pan masala and gutkha consumers. All three cytogenetic end-points showed a statistically significant increase among the habit groups as compared with the controls (30, 31).

Chromosome abnormalities are considered to be one of the most important cytogenetic parameters for the manifestation of genotoxicity. Recently (32) have reported that persons with high frequency of CA develop cancer twice as often as others. During the present investigation pan masala consumers showed significantly increased CA compared with their matched controls. The background frequency of CA (0.86) matched very well with those reported for control individuals in various populations investigated by (33, 34). The present study of chromosomal abnormalities frequency of chromatid type aberrations such as gaps (0.90% *v.* 1.83%) breaks (0.47% *v.* 1.77%), exchanges (0.02% *v.* 0.18) and acentric fragments (0.20% *v.* 0.90%), increased significantly when compared to controls and pan chewers. Isochromatid aberrations also increased significantly such as gaps (0.12% *v.* 0.97%) breaks (0.07% *v.* 0.80%), acentric fragments (0.05% *v.* 0.23%) and dicentrics (0.02% *v.* 0.63%), high percentage of aberrations is found in both chromatid and iso chromatid levels among the pan chewers compared to control group ($P < 0.001$).

In conclusion, the buccal cell micronucleus test is a cost-effective and accurate procedure, which can be easily carried out for population-based studies. MN test is better indicator for genotoxicity damage than SCE and CA.

Furthermore, increased micronuclei frequency in the grossly normal appearing oral mucosa of the high risk individuals is associated with greater risk of oral cancer development as suggested by concept of field carcinogenesis. The present study clearly indicates that pan chewing greatly affect the cells level of micronuclei, sister chromatid exchange and causes chromosomal aberrations such as gaps, breaks, acentric fragments, exchanges, and dicentrics. Therefore, genetic composition of MN, SCE and CA must be studied to determine if they contain specific genes associated with oral carcinogenesis. The results of such studies could have a significant impact on the future use to detect DNA damage in any kind of gonotoxicity and toxicology biomarker.

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