PRINT: ISSN 0972-3757 ONLINE: 2456-6360
Int J Hum Genet, 11(4): 277-282 (2011)
DOI: 10.31901/24566330.2011/11.04.10

Studies on the Genotoxicity of *Gutkha*

Pooja Chadha[1] and Joginder Singh Yadav[2]

[1]Department of Zoology, Guru Nanak Dev University, Amritsar, Punjab, India
[2]Human Genetics Unit, Department of Zoology, Kurukshetra University, Kurukshetra, Haryana, India

KEYWORDS *Gutkha.* Smokeless Tobacco. Chromosome Aberrations. Sister Chromatid Exchanges. Micronucleated Cells

ABSTRACT Nowadays, smokeless tobacco consumption in the form of *gutkha* is receiving more popularity among the youth of India. In the present investigation, cytogenetic studies were carried out on 50 *gutkha* consumers and compared with controls, 50 men who did not have habit of smoking, alcohol drinking and consuming *gutkha*, tobacco and areca nut in any form. Frequency of chromosome aberrations (CA) and sister chromatid exchanges (SCE) were evaluated in peripheral blood lymphocytes, whereas frequency of micronucleated cells (MNC) was evaluated in exfoliated buccal mucosa. A significant increase (p<0.001) was noted in values of all three cytogenetic markers among *gutkha* consumers compared with controls viz. CA (0.92-3.60), SCE (3.66-6.84), MNC (0.09-0.98). The frequency of all end points was higher among high age group individuals (>31) compared with low age group individuals viz CA (1.67-2.81), SCE (4.65-5.80), MNC (0.36-0.70). The increased frequency of these end points was found to be significantly correlated (p<.001) with duration of consumption (b = 0.193, 0.039, 0.032 for CA, SCE and MNC respectively) and number of pouches consumed per day (b being 0.193, 0.441, 0.429 for CA, SCE and MNC respectively). Besides this, significant difference was found between the mean values of CA and SCE among *gutkha* consumers and *gutkha* consumers + drinkers + smokers and also significant increase was found between *gutkha* consumers + smokers and *gutkha* consumers + drinkers + smokers for CA.

INTRODUCTION

Tobacco was introduced in India about 400 years ago. Today a large number of tobacco products are available for human consumption. 40 percent of the tobacco consumed in India is in the smokeless form (*Pan, Pan masala, Zarda, Gutkha*) (Mukherjee and Hadaye 2006). The last two decades have seen a phenomenal growth in the smokeless tobacco industry. The extensive marketing of *gutkha* has led to a widespread addiction amongst school going children. Nair et al. (2004) has estimated that about 5 million young Indians are suffering from oral submucosa fibrosis (a disease which is precursor of oral cancer) as a result of increased popularity of habits of chewing *gutkha* and *pan masala.* Jyoti et al. (2011) have reviewed a large number of studies revealing genotoxicity of *pan masala* and *gutkha.*

Areca nut which constitutes 70-80 percent of *gutkha* contains some specific alkaloids. Arecoline, the most important alkaloid is present in 1 percent dry weight and is found to be genotoxic (Dave et al. 1992). According to IARC (2004), areca nut chewing has been classified to be carcinogenic.

Among flavouring agents, use of synthetic flavours like musk ambrette and musk xylene is well known and the presence of these agents have been detected in the saliva of chewers of betel quid with tobacco and their mutagenicity have been tested in Salmonella/mammalian microsome test (Nair et al. 1986).

Lime, another component of *gutkha* causes local irritation to mucosa and hyperplasia has been observed following application of lime to cheek pouch of hamsters (Dunham et al. 1966). Catechu, another important ingredient contains 2-10 percent catechin (IARC 2004) and has hepatoprotective effects (Lapis et al. 1986). On the other hand, it has been reported that catechu in the presence of lime at an alkaline pH is the most reactive producer of reactive oxygen species (ROS) which are considered to be important in the process of mutagenesis (Nair et al. 1992).

Thus, *gutkha* represents a complex mixture of harmful constituents. Keeping in view the above, it was considered important to look into the genotoxic potential of *gutkha.* The present investigation was carried out on *gutkha* consumers, including those who use *gutkha* only, those who use *gutkha* and smoke, those who use *gutkha* and drink and those who use *gutkha,* drink and smoke as well as on those control individu-

Corresponding author:
Dr. Pooja Chadha,
Lecturer,
Department of Zoology,
Guru Nanak Dev University,
Amritsar, Punjab, India
E-mail: poojachadha77@yahoo.co.in.

als who abstained from all tobacco products. Cytogenetic end points like chromosomal aberrations (CA) and sister chromatid exchanges (SCE) in peripheral lymphocytes and micronucleated buccal mucosa cells (MNC) were evaluated.

MATERIAL AND METHODS

Investigation was carried out on 100 healthy male individuals: 50 controls and 50 *gutkha* consumers. The examined individuals were mostly vegetarians. *Gutkha* consumers were categorized according to their age, number of years of chewing and number of pouches (each pouch contains 2 gm *gutkha* powder) consumed per day, smoking (*bidis* only) and alcohol drinking histories. Most of the volunteers belong to Kurukshetra, Ambala, Karnal and nearby places. Most of them were from lower socio-economic groups being labourers, rickshaw pullers, peons, mess workers and gardeners, not being engaged in any hazardous occupation, that is, they were not exposed to hazardous chemicals at their work places. Attention was paid to exclude any individual who had been subjected to any X-ray treatment or antibiotic therapy three months prior to when samples were taken. Individuals having no history of alcohol use, smoking, tobacco or areca nut chewing in any form were selected as controls. They were matched with exposed individuals having the same socio-economic status, age, gender and diet.

Blood samples were taken using heparinized syringes. Short term lymphocyte cultures were set up within 4 hr of sampling according to the technique of Moorhead et al. (1960) with minor modifications.

Lymphocytes were cultured by adding 0.5 ml of blood in 5 ml of RPMI 1640 medium (Hi media) supplemented with 20% foetal calf serum and 0.1 ml of phytohaemagglutinin (Sigma). Colchicine (10 µg/ml; Sigma) was added to cultures 1 hr. prior to harvesting.

Lymphocytes were harvested after 48 h for assessing chromosomal aberrations (CA). The slides were prepared and stained with 4% Giemsa solution. For each person 100 well spread metaphases were analyzed.

For sister chromatid exchanges (SCE), 5-bromodeoxyuridine (BrdU, 10µg/ml) was added 24h after establishing the cultures. Harvesting was done after 72h. Slides were prepared by the air drying method and stained with Hoechst 33258 (Sigma) and 4% Giemsa solution following the method of Perry and Wolff (1974). For calculating the frequency of SCE per cell, 30 metaphases were analyzed as per standard International norms.

For micronucleus assay, buccal smears on glass slides were transported to laboratory on ice and processed within 3-4 h of sample fixation. The air dried samples were hydrolyzed for 8 min in HCl at 60^0c. After a rinse in tap water and staining in Acetoorecine (2% in 60% acetic acid for 20 min at 40^0c), the samples were given a brief washing in ethanol and distilled water. Counter staining was done with fast green solution with final rinse in ethanol and distilled water. Slides were air dried and coded. At least, 1000 cells were scored and the criteria of Tolbert et al. (1992) were followed for scanning cells for micronuclei.

Two observers, who were unaware of the identity of the sample, in order to remove possible laboratory scoring bias, analyzed the coded samples.

The results were analyzed statistically by two way analysis of variance (Edwards 1971), regression analysis and Mann Whitney U Test (Siegel 1956) using computer software.

RESULTS

Table 1 presents various types of chromosome and chromatid type aberrations among controls and *gutkha* consumers. Total chromosome aberrations among control individuals were 46 and among *gutkha* consumers were 181.

A perusal of Table 2 reveals the mean values of CA, SCE and MNC in controls and *gutkha* consumers and in the lower age group (<31) and the higher age group (>31). Mean values of CA, SCE and MNC in controls were found to be 0.92, 3.66 and 0.09 respectively and in *gutkha* consumers came out to be 3.60, 6.84 and 0.98 respectively. *F* ratio for the effect of *gutkha* consumption on CA, SCE and MNC is 92.15, 1785.13 and 190.18 respectively showing significant effect ($p<.001$) of *gutkha* consumption on these cytogenetic parameters (ANOVA).

Mean values of CA, SCE and MNC in individuals of lower age group (>31) was 1.67, 4.65 and 0.36 respectively and in higher age group (<31) was 2.81, 5.80 and 0.70 respectively. The F ratio for the effect of age on CA is 3.35 showing non-significant effect ($p>.05$) and for SCE

Table 1: Types of chromosomal aberrations in *gutkha* consumers and control individuals

	Control individuals	*Gutkha consumers*
Group		
No. of individuals	50	50
No. of metaphases	5000	5000
Chromosome Type Aberrations		
Dicentrics	0	9 (0.18)
Rings	0	4 (0.08)
Acentric fragments	1 (0.02)	12 (0.24)
Chromosome gaps	4 (0.08)	10 (0.2)
Chromosome breaks	3 (0.06)	12 (0.24)
Translocations	0	6 (0.12)
Total (without gaps)	4 (0.08)	43 (0.86)
Chromatid Type Aberrations		
Gaps	79 (1.58)	177 (3.54)
Breaks	42 (0.84)	136 (2.72)
Isochromatid exchanges	0	2 (0.04)
Total (without gaps)	42 (0.84)	138 (2.72)
Total chromosomal aberrations (without gaps)	46 (0.92)	181 (3.62)

Values in parentheses indicate aberrations per 100 Metaphases

Table 2: Mean Values of CA, SCE and MNC in controls and *gutkha* consumers as well as in lower age group (<31) and higher age group (>31)

Group	*Number of individuals*	*CA*	*SCE*	*MNC*
Control	50	0.92	3.66	0.09
Gutkha consumers	50	3.60[a]	6.84[a]	0.98[a]
Lower age group (<31)	48	1.67	4.65	0.36
Higher age group (>31)	52	2.81	5.80[b]	0.70[b]

a. significantly higher than control value (ANOVA tested), F being 92.15,1785.13,190.08 respectively for CA, SCE and MNC, $p<.001$

b. significantly higher than lower age group (ANOVA tested), F being 27.28,3.83 respectively for SCE and MNC, $p<.001$

Two way interaction (Exposure X Age) is non- significant, F being .046,.797,.287 respectively for CA, SCE and MNC $p>.05$

and MNC is 27.28 and 3.83 respectively ($p < .01$) showing significant effect of age on SCE and MNC. The interactive effect of age X *gutkha* consumption (combined) was found to be non-significant. F ratio for CA, SCE and MNC being .046, .797 and .374 respectively ($p>.05$).

Tables 3 and 4 reveal the frequency of CA, SCE and MNC in controls and *gutkha* consumers with duration of consumption in years and with the number of pouches consumed per day respectively. Linear regression analysis was applied to study the effect of duration of consumption and number of pouches consumed per day on parameters investigated. Value of multiple correlation (multiple R) for dependent variables CA, SCE and MNC came out to be 0.888, 0.750 and 0.822 respectively showing highly significant values ($p <. 001$).

Table 3: Frequency of chromosomal aberrations (CA), sister chromatid exchanges (SCE) and micronucleated buccal mucosal cells (MNC) in controls and *gutkha* consumers with duration of consumption

Duration of consumption (in years)	*Number of samples*	*CA (Mean ± SD)*	*SCE (Mean ± SD)*	*MNC (Mean ± SD)*
Control Individuals	50	0.92 ± 0.77	3.66 ± 0.25	0.09 ± 0.08
Gutkha Consumers	50	3.60 ± 1.82	6.84 ± 0.54	0.98 ± 0.45
0-5	6	1.83 ± 1.47	6.35 ± 0.58	0.78 ± 0.35
6-10	19	2.84 ± 1.34	6.73 ± 0.45	0.83 ± 0.38
11-15	12	3.75 ± 1.35	6.79 ± 0.63	1.03 ± 0.36
16-20	6	4.16 ± 0.98	7.21 ± 0.24	1.05 ± 0.53
21-25	5	6.00 ± 1.00	7.30 ± 0.24	1.46 ± 0.43
26-30	2	7.50 ± 0.70	7.34 ± 0.02	1.90 ± 0.14
Regression analysis		R=0.888, R^2=0.789, F=88.063, p<.001 b=0.193, t=9.900, p<.001	R=0.750, R^2=0.562 F=30.201, p<.001 b=0.039, t =4.763, p<.001	R=0.822, R^2=0.675 F=30.201, p<.001 b=0.032, t=5.493, p<.001

Table 4: Frequency of chromosomal aberrations (CA), sister chromatid exchanges(SCE) and micronucleated buccal mucosal cells(MNC) in controls and gutkha consumers with number of pouches consumed per day

Number of pouches consumed per day	*Number of samples*	*CA (Mean ± SD)*	*SCE (Mean ± SD)*	*MNC (Mean ± SD)*
Controls	50	0.92 ± 0.77	3.66 ± 0.25	0.09 ± 0.08
Gutkha consumers	50	3.60 ± 1.82	6.84 ± 0.54	0.98 ± 0.45
6-10	8	1.75 ± 0.88	6.20 ± 0.56	0.50 ± 0.20
11-15	10	3.00 ± 1.49	6.82 ± 0.47	0.84 ± 0.32
16-20	14	3.71 ± 1.93	6.85 ± 0.53	0.99 ± 0.48
21-25	9	4.44 ± 1.74	6.97 ± 0.33	1.24 ± 0.35
26-30	9	4.88 ± 1.26	7.28 ± 0.21	1.41 ± 0.31
Regression analysis		R=0.888, R^2=0.789 F=88.063, p<.001 b=0.193, t=9.90, p<.001	R=.750, R^2=0.562 F=30.201, p<.001 b=0.441, t=5.595, p<.001	R=0.822, R^2=0.675 F=48.868, p<.001 b=0.429, t=7.583, p<.001

The value of R^2 (which determines the proportion of variance in parameters studied jointly determined by variation in duration of consump-

tion and number of pouches consumed per day) came out to be 0.789, 0.562 and 0.675 respectively. The value of regression coefficient, that is, *b* for duration of consumption was 0.193, 0.039, 0.032 respectively ($p < .001$) for CA, SCE and MNC showing a linear influence of duration of consumption on SCE, CA and MNC. The values of *b* for number of pouches consumed per day for CA, SCE and MNC being 0.193, 0.441 and 0.675 respectively ($p < .001$) showed significant positive influence of the number of pouches consumed per day on the parameters considered .

Gutkha consumers were divided into different subgroups according to their smoking and alcohol-drinking pattern (Table 5). Mann Whitney U Test (Siegel 1956) was applied to study any significant difference in the value of parameters showed in different subgroups. Significant difference was found between subgroup 1 and 4 of only *gutkha* consumers and *gutkha* consumers and smokers + drinkers for CA and SCE, *U* being 44 and 46.5 ($p < .05$) for CA and SCE respectively.

Table 5: Frequency of chromosomal aberrations (CA), sister chromatid exchanges (SCE) and micronucleated buccal mucosal cells (MNC) in different subgroups of *gutkha* consumers.

Groups	*Number of individuals*	*CA (Mean± SD)*	*SCE (Mean± SD)*	*MNC (Mean± SD)*
Control	50	0.92 ±0.07	3.66 ± 0.25	0.09 ± 0.08
Total *Gutkha* Consumers	50	3.60 ± 1.82	6.84 ± 0.54	0.98 ± 0.45
Subgroups :				
1) Only *Gutkha* Consumers	22	2.95 ± 1.55	6.69 ± 0.55	0.86 ± 0.35
2) *Gutkha* Consumers+ Drinkers	12	4.16 ± 1.80	6.85 ± 0.65	1.10 ± 0.43
3) *Gutkha* Consumers+ Smokers	7	2.85 ± 1.06	6.87 ± 0.38	0.85 ± 0.49
4) *Gutkha* Consumers+ Drinkers+ Smokers	9	5.00 ± 2.12[a,b]	7.16 ± 0.34[a]	1.21 ± 0.57

a. Significantly higher than subgroup 1 of only *Gutkha* Consumers.(Mann Whitney *U* Test,*U*=44, and 46.5, $p<.05$ for CA and SCE respectively)

b. Significantly higher than subgroup 3 of *Gutkha* Consumers+Smokers (Mann Whitney *U* Test, *U*=13.5 , $p<.05$)

DISCUSSION

There is a great need to examine the possible genotoxic effects of smokeless tobacco used in the form of *gutkha* (*Pan masala* with tobacco) which contains all ingredients of traditional betel quid except betel leaf. Chemo preventive effect of betel leaf on the genotoxicity of *pan masala* has been reported (Trivedi et al. 1994). There is possibility of increased oral submucosa fibrosis epidemic in near future. Anila et al. (2011) revealed an increase in micronuclei in oral sub mucosa fibrosis patients as compared to healthy individuals and emphasized that *gutkha* chewing habit in the younger age increased the chances of malignant transformations. Factors, which may be responsible, are the addition of tobacco content, the absence of the betel leaf and its carotenes and the much higher dry weight of *pan masala/gutkha* (Babu et al. 1996). Metaphase analysis of CA frequency has been widely used for identifying the clastogenic compounds (Kliesch and Alder 1983) and the misrepaired chromosomal lesions, which escape being detected as CA, can be detected effectively by studying SCE frequencies. Stich (1986), and Stich et al. (1990) have widely used the micronuclei assay at a population level for studying the effects of tobacco/areca nut chewing habits on buccal mucosa. Increase in the frequency of SCE has been reported in cells of individuals exposed to various mutagens as in cigarette and *bidi* smokers (Ghosh and Ghosh 1987; Husum et al. 1986; Yadav and Thakur 2000; Yadav et al. 2001) and in betel and tobacco chewers (Ghosh and Ghosh 1984). Increased frequencies of micronucleated cells in exfoliated buccal mucosa and high incidence of CA and SCE in peripheral lymphocytes have been observed in users of *pan masala* with and without tobacco in comparison with control individuals (Dave et al. 1991; Trivedi 1992; Yadav and Chadha 2002; Beena and Patel 2009). A dose dependent significant increase in frequency of chromosomal aberrations and micronuclei was observed in bone marrow cells of mice after treatment with *pan masala* and *gutkha* (Mojidra et al. 2009). Using in vitro short term assay, the dimethyl sulphoxide (DMSO) extract of *pan masala* with and without tobacco has been found to increase the frequency of CA, SCE and micronucleated cells in cultures without metabolic activation (Patel et al. 1994). Besides this, aque-

ous extract of *pan masala* when used in vitro significantly increased the frequency of CA and SCE in Chinese hamster ovary cells in dose dependent manner (Patel et al. 1994; Adhyaryu et al. 1989). Similarly, increase in frequency of micronucleated buccal mucosa cells in individuals consuming *pan masala* and *gutkha* were observed by Gandhi and Kaur (2000), Siddique et al.(2008)and Fareed et al. (2011).

During the present investigation, a significant increase in the frequency of CA, SCE and MNC has been observed in individuals consuming *gutkha* when compared with controls. Besides, this investigation has revealed a significant positive correlation of duration of consumption and frequency of daily use with all the end points studied viz. CA, SCE and MN. Significant positive correlation between frequency of micronuclei in buccal mucosa and consumption of tobacco was earlier reported by (Sarto et al. 1987). The role of age in determining these end points was also studied during the present investigation. Mean values of SCE and MNC are significantly higher in higher age group individuals and mean value of CA is also greater in higher age group than lower age group but not significantly. As such, age plays an important role in determining these cytogenetic markers. Similarly, role of the age on the frequency of spontaneous micronucleus formation was earlier reported (Tice and Stelow 1985; Fenech and Morely 1985; Ghosh et al. 1990). Earlier studies by Yadav and Chadha (2002) on *pan masala* (without tobacco) also showed significant effect of age on all these end points. The values of CA and SCE showed significant increase in case of those *gutkha* consumers who also smoked and drank. These parameters showed higher values even when compared with *gutkha* consumers who smoked. Thus, drinking increase the genotoxic potential of *gutkha*. Genotoxic potential of drinking alcohol was earlier reported (Patel et al. 1994).

Although enough care was taken for matching the exposed and the controls, it was not possible to match them 100%, for example, the food they take and the particular habitat in which they live. Some of them may be forced to be passive smokers and occasional drinkers. Even then we find that the consumption of *gutkha* (*pan masala* containing tobacco) in association with smoking and drinking caused sufficient damage to genetic material and thus has the potential to cause cancer. So there is a dire need to put ban on the manufacture and sale of such products and the acceleration of the programmes which are aimed at reduction or elimination of use of these products.

ACKNOWLEDGMENTS

Research supported by Kurukshetra University, Kurukshetra. The authors sincerely thank Dr. C.R. Darolia for his kind help in statistical analysis of the results and to Dr. A.S. Yadav for varied help.

REFERENCES

Adhvaryu SG, Dave BJ, Trivedi AH 1989. An *in vitro* assessment of the genotoxic potential of *pan masala*. *Indian J of Medical Res,* 90: 131-134.

Anila K, Kaveri H, Nailkmasur VG 2011. Comparative study of oral micronucleated cell frequency in oral submucosa fibrosis patients and healthy individuals. *J Clin Exp Dent,* 3(3): 201-206.

Babu S, Bhat RV, Kumar PU, Sesikaran B, Rao KV, Aruna P, Reddy P, Reddy R et al. 1996 . A comparative clinicopathological study of oral submucosa fibrosis in habitual chewers of *pan masala* and betel quid. *Clinical Toxicol,* 34(3): 317.

Beena, Patel P 2009. Micronucleus and chromosomal aberrations in healthy tobacco chewers and controls: A study from Gujarat, India. *Arch Oncol,* 17: 7-10.

Dave BJ, Trivedi AH, Adhvaryu SG 1992. *In vitro* genotoxic effects of areca nut extract and arecoline. *J Cancer Res Clin Oncol*, 118: 283-288.

Dave BJ, Trivedi AH, Adhvaryu SG 1991. Cytogenetic studies reveal increased genomic damage among *pan masala* consumers. *Mutagenesis,* 6: 159-164.

Dunham LJ, Muir CS, Hamner JJE 1966. 111, Epithelial atypia in hamster cheek pouches treated repeatedly with calcium hydroxide. *British J of Cancer,* 20: 588-593.

Edwards AL 1971. *Experimental Design in Psychological Research*. New York : Holt.

Fenech M, Morely AA 1985. The effect of spontaneous and induced micronuclei. *Mut Res,* 148: 99-105.

Freed M, Afzal M, Siddique YH 2011. Micronucleus investigation in buccal mucosa cells among *pan masala/gutkha* chewers and its relevance for oral cancer. *Bio and Med,* 3(2): 8-15.

Gandhi G, Kaur R 2000. Cytogenetic studies in exfoliated cells of high cancer risk groups, *pan masala* chewers. *Human Ecololgy Special Issue,* 9: 221-228.

Ghosh BB, Talukder G, Sharma A 1990. Frequency of micronuclei induced in peripheral lymphocytes of trimethylin chloride. *Mut Res,* 245: 33-39.

Ghosh R, Ghosh PK 1984. Sister chromatid exchanges in betel and tobacco chewers. *Mut Res,* 139: 79-81.

Ghosh R, Ghosh PK 1987. The effect of tobacco smoking on the frequency of sister chromatid exchanges in human lymphocyte chromosomes. *Cancer Genet Cytogenet,* 27: 15-19.

Husum B, Wulf HC, Niebuhr E 1986. Sister chromatid exchange frequency correlates with age, sex and

cigarette smoking in a 5-year material of 533 healthy adults. *Hereditas,* 105: 17-21.

IARC2004. *Monographs on the Evaluation of the Carcinogenic Risks to Humans, Tobacco Habits Other than Smoking; Betel Quid and Areca Nut Chewing*. Volume 85. Lyon : International Agency for Research on Cancer.

Jyoti S, Afzal M, Siddique Y H 2011. Genotoxic effects of *pan masala* and *gutkha*: A review. *World J Zool,* 6(3): 301-306.

Kliesch U, Alder ID 1983. Chromosome analysis and micronucleus test in mouse bone marrow are not equally sensitive as shown by results with 5 chemical mutagens. *Mut Res,* 113: 340-346.

Lapis K, Jeney A, Divald A, Vajta G, Zalatnai A, Schaff Z et al. 1986. Experimental studies on the effect of hepatoprotective compounds. *Tokai J of Exp and Clin Medicine,* 11: 135-145.

Moorhead PS, Nowel PC, Mellamn WJ, Battips DM, Hungerford DA et al. 1960. Chromosome preparations of leukocytes cultured from human peripheral blood. *Experimental Cell Res,* 20: 613-616.

Mojidra BN, Archana K, Gautam AK, Verma Y, Lakkad BC et al. 2009. Evaluation of genotoxicity of *pan masala* employing chromosomal aberration and micronucleus assay in bone marrow cells of the mice. *Toxicol and Health,* 25(7): 467-471.

Mukherjee K, Hadaye RS 2006. *Gutkha* consumption and its determinants among secondary school male students. *Indian J of Commun Medicine,* 31(3): 177.

Nair J, Oshima H, Malaveille C, Friesen MO, Nerlhl K, Hautefeuilie A Bartsch H et al. 1986. Identification, occurrence and mutagenicity in *Salmonella typhimurium* of two synthetic nitroarenes, musk ambrette and musk xylene, in Indian chewing tobacco and betel quid. *Food Chem Toxicol,* 24: 27-31.

Nair UJ, Obe G, Friesen M, Goldberg MT, Bartsch H et al. 1992. Role of lime in the generation of reactive oxyzen species from betel quid ingredients. *Envt Health Prospectives,* 98: 203-205.

Nair U, Bartsch H, Nair J 2004. Alert for an epidemic of oral cancer due to use of betel quid substitutes *gutkha* and *pan masala:* A review of agents and causative mechanisms. *Mutagenesis,* 19(4): 251-262.

Panigrahi GB, Rao AR 1982. Chromosome breaking ability of arecoline: A major betel nut alkaloid in mouse bone marrow cells *in vivo*. *Mut Res,* 103: 197-204.

Patel RK, Jaju RJ, Bakshi SR, Trivedi AH, Dave BJ, Adhvaryu SG et al. 1994. *Pan masala*- a genotoxic menace. *Mut Res,* 320: 245.

Patel RK, Trivedi AH, Jaju RJ, Adhvaryu AG, Balar DB et al. 1994. Ethanol potentiates the clastogenicity of *pan masala* an *in vitro* experience. *Carcinogenesis,* 15(8): 2017-2021.

Perry P, Wolf S 1974. New giemsa method for the differential staining of sister chromatids. *Nature,* 251: 156-158.

Sarto F, Finotto S, Giacomelli L, Mazzotti D, Tomanin R, Levis AG et al. 1987. The micronucleus assay in exfoliated cells of human buccal mucosa. *Mutagenesis,* 2: 11-17.

Siddique YH, Ara G, Beg T, Afzal M 2008. Micronucleus investigation in oral mucosa cells of *gutkha/ pan masala* chewers. *Indian Biologist,* 40: 1-8.

Siegel S 1956. *Non- parametric Statistics for Behavioural Sciences*. Kogakusha: Mcgraw Hill.

Stich HF 1986. The use of micronuclei in tracing the genotoxic damage in oral mucosa of tobacco users. In : D Hoffman, CC Harris (Eds.): *Mechanisms in Tobacco Carcionogenesis*. New York: Banbury Report No. 23, Cold Spring Harbor Laboratory, pp. 99-111.

Stich HF, Acton AB, Palcic B 1990. Towards an automated micronucleus assay as an internal dosimeter for carcinogen – exposed human population groups. In : P Band (Ed.): *Recent Results in Cancer Research, Occupational Cancer Epidemiology.* Berlin Heidelberg, New York : Springer Verlag, Vol. 120: 94-105.

Tice RR, Stelow RB 1985. DNA repair and replication in aging organisms and cells. In : C E Finch, E L Schneirder (Eds.): *Handbook of Biology of Aging.* 2nd Edition. New York : Van Nostrand Reinhold, pp. 173-224 .

Tolbert PE, Shy CM, Allen J 1992. Micronuclei and other nuclear anomalies in buccal smears: Method development. *Mut Res,* 271: 69-77.

Trivedi AH 1992. *A Study on Carcinogenic Potentials of Tobacco.* Ph.D. Thesis. M.S. University of Baroda, Baroda, India, pp. 148-179.

Trivedi AH, Dave BJ, Adhvaryu SG 1993. Monitoring of smokeless tobacco consumers using cytogenetic end points. *Anticancer Res,* 13: 2245-2249.

Trivedi AH, Patel RK, Rawal UM, Adhvaryu SG, Balar DB et al. 1994. Evaluation of chemopreventive effects of betel leaf on the genotoxicity of *pan masala. Neoplasma,* 41(3): 177-181.

Yadav JS, Chadha P 2002. Genotoxic studies in *pan masala* chewers: A high cancer risk group. *Int J Human Genet,* 2 (2): 107-112.

Yadav JS, Thakur S 2000. Cytogenetic damage in *bidi* smokers. *Nicotine and Tobacco Res,* 2: 97-103.

Yadav JS, Thakur S, Chillar AK 2001. Genetic risk assessment in cigarette smokers. *Human Ecology in the New Millenium,* 10: 165-170.