Evaluation of Genetic Damage in Tobacco Chewing Population by In-vitro SCE Assay: a Review from Coastal Andhra Population

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Abstract

Background/Objectives: To understand the genotoxicity and cytotoxicity potentials in tobacco-chewing habitues from coastal Andhra Population. Place and Duration of Study: Translational Research Institute of Molecular Sciences, between October 2013 to October 2014. **Methods/Statistical Analysis:** Present study was carried out using 35 individuals (28 men, 7 women; age range 25-56 years) who are having a habit of chewing tobacco from 5-20 years were tested along with 35 controls with same age group. Samples were cultured and stained with Hoechst 3325. Scored chromosomal abnormalities, SCE's, CPK and MI using statistical evaluations. **Results/Findings**: In this study we have scored the numerical and structural abnormalities, Mitotic Index (MI), Cell Proliferation Kinetics (CPK) and Sister Chromatid Exchanges (SCE's) of the presented samples have been calculated. Although significant numerical aberration observed, structural abnormalities were notably seen in a few cases. When compared with controls tobacco-chewing habitués does increase induced significant mito depression. Our data reveals that samples CPK values were remarkably decreased when compared to control value. **Conclusion/Application:** The demonstration of chromosomal damage in lymphocytes from tobacco chewing habitues strongly indicates that tobacco should be categorized as a human carcinogen and major risk factor for oral, throat and pharynx cancers.

Keywords: BrdU-labelling, CA, CPK, MI, SCE

1. Introduction

Smokeless tobacco is the cheapest, least taxed and most commonly used tobacco products in India. They are highly addictive and high in carcinogens. They cause a broad spectrum of diseases; yet awareness about their ill-effects is low. Smokeless tobacco products containing areca nut, e.g. gutka and mawa, are especially addictive and carcinogenic. Smokeless tobacco is used in almost one-third of households in rural areas whereas almost one-sixth of households in urban areas. Smokeless Tobacco may refer to Dipping tobacco (a type of tobacco that is placed between the lower or upper lip and gums), Chewing Tobacco (a type of tobacco that is chewed),

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Snuff (a type of tobacco that is inhaled or "snuffed" through the nose), Snus (a Swedish product similar to dipping tobacco), Creamy snuff (a fluid tobacco mixture marketed as a dental hygiene aid, albeit used for recreation). Chewing tobacco increases the risk of oral cancers, throat and pharynx cancers¹⁻³. Chemicals have been recognized as etiologic factors in human carcinogenesis since the 18th century observations of the scrotal carcinogenicity of soot in chimney sweepers by Sir Percival Pott⁴. Exposure to environmental carcinogens can induce DNA lesions; elicit infidelity of DNA repair, and cause the instability phenomenon, and subsequent consequences as e.g., chromosomal breakage syndromes are since

the environmental chemical exposure level continuously increases with the increasing number of existing and commercially available chemicals⁶. Tobacco has addictive properties due to the presence of nicotine which causes signs of dependence of tolerance in the consuming adult. The extent and increasingly speed of the tobacco addictions are believed to be directly proportional to the biological strength of nicotine addiction. Follow-up investigation of the frequency of structural and numerical Chromosome Aberrations (CA) and Sister-Chromatid Exchanges (SCE) in peripheral blood lymphocytes of Costal Andhra tobacco chewing habitues (Gutkha and Pan) and control population with same age group.

2. Material and Methods

2.1 Materials

RPMI 1640 (GIBCO), PHA-phytohemagglutinin (GIBCO), Colchicine (Sigma), Mytomycin C, 0.75M KCl (Qualigens), Methanol (Qualigens), Glacial acetic acid (Qualigens), BrdU (Sigma), Hoechst 3325 (Sigma), Giemsa's stain, Sorenson's buffer, 2XSSC, Sterile slides, 15mL conical bottom centrifuge tubes and glass droppers.

2.2 Methods

Sodium heparinized peripheral blood samples were collected from 35 individuals (28 men (80%) and 7 women (20%), 25-55 age group) who are having a habit of chewing tobacco from 5-20 years were tested along with 35 controls consisted of 28 healthy male (80%) and female (20%) aged 25-55 years with no history of exposure to clastogenic and/or aneugenic agents and of socio-economic level also similar to that of the experimental subjects. At the time of blood collection (3 ml/individual), the workers signed a term of informed consent and replied to a questionnaire elaborated to determine the profile and habits of the study population. The study procedures used in the present study were approved by the local ethical committee. Peripheral blood samples were cultured using standard protocol. Sodium heparinized peripheral blood samples were cultured using RPMI 1640 complete medium, a mitogen phytohemagglutinin (PHA) and L-Glutamine. Cultures were Incubated in 37°C incubator for 72 hrs. At the end of the incubation the spindle fibre formation was arrested using colchicine an alkaloid was added and incubated for 30-40 min. centrifuged at 800g for 10 min. Hypotonic treatment was given with 0.75M KCl and incubated for 15-20 min. Centrifuged at 800g for 10 minutes. Fixative (3:1 ratio of methanol and acetic acid) washes were given for 4-5 times. Prepared slides and scored MI in 1000 cells, CPK in 150 cells. Sister Chromatid Exchange assay was performed using the protocol of Anderson D^{7,8} and P.E Perry⁹⁻¹¹ with some changes.

2.2.1 Microscopic Evaluation

2.2.1.1 Chromosomal Aberrations (CA)

Chromatid and chromosomal breaks and fragments were the most frequent chromosomal aberrations found. Results were expressed as percentage of aberrant cells. Scoring of the all parameters including MI, CPK and SCE, is done using Upright Light Microscope as follows.

2.2.1.2 Sister Chromatid Exchanges (SCE)

The frequency of SCE was observed in 35 differentially stained metaphases The SCE frequency including exchanges in the centromere was counted in M2 metaphases.

2.2.1.3 Mitotic Index (MI)

MI is a measure for the proliferation status of a cell population. It is defined as the ratio between the number of cells in mitosis and the total number of cells. Mitotic index was calculated by using the formula:

No. of dividing cells Mitotic index = X 100 Total no. of cells

2.2.1.4 Cell Proliferation Kinetics (CPK)

Cell-cycle specific patterns were determined by Ml, M2, and M3 metaphases.

CPK= M1+2M2+3M3

100

2.2.1.5 Statistical Analysis

Repeated measures of Analysis of Variance (ANOVA) were applied in the experiments performed for MI, CPK, CA and SCE.

3. Results

The present study involves 35 samples of tobacco chewing population, have been tested for genotoxic and cytotoxic effects with 35 sample with same age groups from costal Andhra population. In this study we have studied the numerical and structural abnormalities of chromosomes have been scored (Figure 1). And also Mitotic Index (MI), Cell Proliferation Kinetics (CPK) and Sister Chromatid Exchanges (SCE's) of the participating samples have been calculated (Figure 2). We observed more number of SCE's in group A (chromosome 1 & 3), B(chromosome 4 & 5), C(chromosome 11&12), E(chromosome 17) and G(chromosome 22). These SCE's frequencies are significantly different from the control population (Table 1). No significant numerical aberration has been observed. But structural abnormalities are very significantly seen in few cases. Genotoxicology monitoring including cytogenetic investigations has been performed in several control, and tobacco chewing human population as it is summarized on Table including the control data.

Table shows Mitotic Index, CPK and SCE's of cultures from the samples of tobacco chewing population. The mean value of MI of control is 5.1103 whereas samples Mitotic Index are 2.3089. When compared to control values tobacco chewing habitues samples does increase induced significant mito depression. The CPK value of control group is 0.4360 whereas sample's CPK value is 0.2880. Data reveals that sample CPK value was significantly decreased when compared to control value.

Table 1. Mitotic index, CPK and SCE's of cultures

Exchange frequency in control is 5.5074, whereas tobacco chewing population 20.4309 SCE's per metaphase on an average.



Figure 1. Chromosomal breaks and gaps.



Figure 2. The metaphase from the Sample-3 showing more no. of SCEs and gaps.

CATEGORY	NUMBER OF SAMPLES	NUMBER OF METAPHASES SCORED/SAMPLE	МІ	СРК	SCE/CELL Mean ± S.E
Controls	35	150	5.1103 +/- 0.53567	0.4360 +/- 0.0359	5.5074 <u>+</u> 0.65919
MNC Induced	10	150	2.0021+/- 0.5213	0.1875+/- 0.0034	23.35 ± 0.38*
Tobacco chewing habitués	35	150	2.3089 +/- 0.5163	0.2880+/-0.0609	20.4309 <u>+</u> 2.3125*

P<0.01*

Significant number of Sister Chromatid Exchanges (SCE's) in tobacco chewing population cultures when compared to control culture (Figure 3). The mean Sister Chromatid Figure 3 showed more number of Sister Chromatid Exchanges in A (chromosome1 & 3) group, B (chromosome 4 & 5), C (chromosome 11&12),



Figure 3. Exchanges in Sister Chromatids.

E (chromosome 17) and G (chromosome 22) groups. These SCE's frequencies are significantly different from the control population.

4. Discussion

In our study we have received surprising results. We found structural abnormalities, but no detectable levels of numerical abnormality. Table reveals the genetic damage in tobacco chewing habitués. Chemical constituents in the tobacco leaf such as NNK and PAHs require metabolic activation to exert their carcinogenic effects; there are competing detoxification pathways, and the balance between metabolic activation and detoxification differs among individuals and will affect cancer risk¹²⁻¹⁴. Statistical analysis indicates there is marginal increase or significant increase in chromosomal abnormality rates in observations. Significant number of Sister Chromatid Exchanges (SCE's) in tobacco chewing population cultures when compared to control culture. Chewing tobacco is highly addictive. In the duration of a half hour chew, the average smokeless tobacco user ingests an amount of nicotine which is equivalent to the amount in 4 cigarettes Chamberlain¹⁵. It would take nearly 60 cigarettes to equal the amount of nicotine in a single can of chewing tobacco. Our findings may indicate an emerging public health problem, since our subjects were young and adult have lesions that could be signs for increased risk of developing oral malignancies^{16,17}.

5. Conclusion

Various studies showed that the tobacco chewing and tobacco smoking effects on bio molecule damage. In the present context we have under taken the study to assess the DNA damage using 35 peripheral blood samples from tobacco (Gutkha or Pan Paragh etc.) chewing habitues for in vitro cultures. The present study provides the reproducible evidence regarding genotoxicity and cytotoxicity of tobacco consumption. The demonstration of chromosomal damage in lymphocytes from tobacco chewing habitues strongly indicates that tobacco should be categorized as a human carcinogen and major risk factor for oral cancer and throat and pharynx cancers. In view of these findings, the present study indicates that tobacco users should be considered a high risk group and need to be monitored for health hazards including cancer.

6. References

- 1. Andersson G, Axell T. Clinical appearance of lesions associated with the use of loose and portion-bag packed Swedish moist snuff: a comparative study. J Oral Pathology. 1989; 18:2–7.
- Albertini RJ, O'Neill JP, Ballinger SW, Nicklas JA. Biomarkers of human genetic risk. In: Fortner JG, Sharp PA, editors. Accomplishments in cancer research. 1995; 99–114.
- Ames BN. Cell proliferation as a major risk factor for cancer: a concept of doubtful validity. Cancer Res. 1996; 55:3759-62, Correspondence re: Farber E. Cancer Res. 1995; 56:4267-9.
- Au WW. Abnormal chromosome repair and risk of developing cancer. Env Hlth Perspect. 1993; 101(Suppl. 3):303-8.
- 5. IARC, International Agency for Research on Cancer. Cancer causes, occurrence and control. In: Tomatis L, editor. IARC Monographs No. 100. Lyon: IARC; 1990.
- 6. WHO. World Health Statistics Annuals 1984-88. Geneva: World Health Organisation; 1988.
- Anderson D, Jenkins PC, Dewdney RS, Francis AJ, Godberg P, Butterworth KR. Chromosome aberrations, mitogen induced blastogenesis and proliferative rate index in peripheral lymphocytes from 106 control individuals of the U.K. population. Mutat Res. 1988; 204:407–20.
- Anderson D, Francis AJ, Godbert P, Jenkinson PC, Butterworth KR. Variability in chromosome aberrations, sister chromatid exchanges, and mitogen-induced blastogenesis in peripheral lymphocytes from control individuals. Env Hlth Perspect. 1993; (Suppl 3):83–8.

- Perry PE, Henderson L, Kirkland D. Sister chromatid exchange in cultured cells. Report of the UKEMS Subcommittee on Guidelines for Mutagenicity Testing, Part II. 1984; 17:89–122.
- Perry PE, Thomson EJ. The methodology of sister chromatid exchange. In: Kilbey BJ, Legator M, Nichols W, Ramel C, editors. Hand book of Mutagenicity Test Procedures. 2nd Ed. Amsterdam: Elsevier Scientific; 1984; 34:495–530.
- Perry PE. Chemical mutagens and sister chromatid exchange. In: de Serres FJ, Hollaender A, editors. Chemical mutagens. New York: Plenum Publishing Co; 1980; 6:1–39.
- Brunnemann KD, Genoble L, Hoffmann D. N-nitrosamines in chewing tobacco: an international comparison. J Agric Food Chem. 1985; 33:1178–81.

- Brunnemann KD, Rivenson A, Adams JD, Hecht SS, Hoffmann D. A study of snuff carcinogenesis. Lyon, France: IARC Scientific Publications; 1987; 84:456–9.
- Brunnemann KD, Qi J, Hoffmann D. Chemical profile of two types of oral snuff tobacco. Food Chem Toxicol. 2002; 40:1699–703.
- 15. Alhadeff WJB. Current protocols in human genetics. 1994; 8(6):1-8.
- Hoffmann D, Hoffmann I. The changing cigarette. J Toxicol Environ Health. 1997; 50:307–64.
- Hoffmann D, Hecht SS. Advances in tobacco carcinogenesis. In: Cooper CS, Grover PL, editors. Handbook of experimental pharmacology. Heidelberg (Germany): Springer-Verlag; 1990; 94:63–102.