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ORIGINAL ARTICLE

Cytogenetical analysis in blood lymphocytes of cigarette smokers in Tiruchirappalli district, Tamil Nadu, India

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KEYWORDS

Chromatid aberration;
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Abstract *Background:* Tobacco smoke causes serious health ill effects to human population. Cancer and cardiovascular diseases are more common in smoking subjects.

Aim: The present study is focused about the genetical changes in smoking subjects based on their age and pack years.

Subjects and methods: Based on a survey report, 160 subjects are selected from Tiruchirappalli district, Tamil Nadu, India. Venous blood and buccal smear samples are collected from each subject.

Results: Increased CA is observed in heavy smokers compared to light and non smokers which is 8.90 ± 2.58, 4.58 ± 2.36 and 4.31 ± 1.17, respectively. Both medium and light smokers showed significantly increased CA frequencies than control. Comet assay showed increased percentage of abnormalities in smokers (light, medium and heavy) than non-smokers.

Conclusion: The frequencies of MN in buccal epithelial and blood lymphocytes are high in smokers; particularly heavy smoker group showed significantly increased results. Among them, the lymphocytic cells showed high MN frequency.

Abbreviations: %TL, percentage of tail length; ANOVA, analysis of variance; CAs, chromosome aberrations; CBMN, cytokinesis-block micronuclei; CTAs, chromatid type aberrations; DNA, deoxyribonucleic acid; DSB, double strand break; EDTA, ethylene diamine tetra acetic acid; PAHs, polycyclic aromatic hydrocarbons; KCl, potassium chloride; MN, micronuclei; MTL, mean tail length; MTM, mean tail movement; NaCl, sodium chloride; PBLs, peripheral blood lymphocytes; SCGE, single cell gel electrophoresis; SPSS, statistical package for social studies; TCA, total chromosome aberrations

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1. Introduction

Smoking is a major cause for cancer, cardiovascular diseases and chronic obstructive pulmonary diseases ([1,2]). Cigarette smoke constitutes approximately 5311 chemical compounds [3] including over 50 known carcinogens such as polycyclic aromatic hydrocarbons (PAHs), *N*-nitrosamines, aromatic amines, and trace metals [4], which act as important mutagenic factors which cause damages to human genetic material [5].

Tobacco smoke induces an array of genetic aberrations including gene mutations, chromosome aberrations (CAs), sister chromatid exchanges and DNA strand breaks [6]. Chromosome aberrations and micronuclei (MN) frequency studies are considered as cytogenetic endpoints and act as sensitive parameters for assessing genotoxic effects of chemical or physical mutagens [7]. Chromosomal aberrations are the important biological consequences of human exposure [8] having a firm place in screening strategies for mutagenic/carcinogenic agents ([9,10]).

Structural chromosomal aberrations in peripheral blood lymphocytes (PBLs) have been applied as a biomarker of early effects of genotoxic carcinogens [11] and usually divided into chromosome-type aberrations (CSAs) and chromatid-type aberrations (CTAs), with different mechanisms of formation [12]. CA studies also act as a biomarker of health outcome which measures the genetic damage due to exposure of various mutagens and probably the only one which has been internationally standardized and validated ([11,13]). DNA double strand breaks (DSBs) are the principal lesions in the process of CA formation ([14,15]).

Micronuclei (MN) are small, extranuclear bodies that arise in dividing cells from acentric chromosome/chromatid fragments or whole chromosomes/chromatids. It is primarily formed due to chromosome segregation machinery defects such as deficiencies in the cell cycle controlling genes, failure of the mitotic spindle, kinetochore or other parts of the mitotic apparatus or by damage to chromosomal substructures and mechanical disruption [11].

Comet assay or single cell gel electrophoresis (SCGE) is a rapid, simple, and sensitive technique for measuring and analyzing DNA breakage in individual cells ([16,17]) and extensively used in human biomonitoring [18]. In Comet assay, the intensity of DNA damage is assessed by computing the tail moment by three tail parameters (tail length, tail intensity and tail moment) [19]. The present study is framed to ascertain the cytogenetical damages in buccal epithelial cells and peripheral blood lymphocytes by evaluating the frequencies of CAs, CTs and MN due to the inhalation of tobacco (cigarette) smoke in the smokers.

2. Subjects and methods

2.1. Subject selection and sample collection

The subjects ($n = 80$) are selected based on pedigree analysis, smoking duration and an initial short survey in self report format which integrated age, duration of cigarette smoking, hereditary problem, medical status and facts about their profession. Subjects are the local residents of Tiruchirappalli district in Tamil Nadu, India. The foremost inclusion criteria in the present study embrace the analysis of pack years (No. of packs of cig. smoked/day × duration of cigarette smoking in years) [20], life style factors (alcohol intake and smoking) and age. The exclusion criteria included the elimination of subjects from viral infection, occupational history, exposure to radiation and chemicals, surgery, chemotherapy, autoimmune diseases, immunology, and genetic disorders. All the controls (non-smokers) are physically and mentally normal subjects who had no history of any genetical disorders.

The study is conducted according to the Institutional Human Ethical clearance and Helsinki [21] procedure. Informed consents are obtained from both smoker and non-smoker subjects. The smokers (exposed subjects) and the non-smokers (controls) are divided into two groups based on their age (Group I: 20–33 years, Group II: 34–50 years). Based on their comparison of age and pack years, smokers are classified into three groups as light smokers ($\leqslant$15 years), medium smokers (16–25 years) and heavy smokers ($>$25 years). Venous blood (5 ml) samples are collected [22] and transferred into an EDTA containers from each subject (separately).

2.2. Micronuclei assay in buccal epithelial cells

The Subjects are instructed to wash their mouth with sterile water. Buccal cells are collected by gentle scrapping of wooden spatula on their cheek. The spatula is stored in saline and centrifuged at 8000 rpm for 5 min. The cell pellet are collected and fixed in methanol:acetic acid (3:1) solution. The fixed cells onto a slide are air dried and stained with Felugen: Fast Green stain and observed under Leica Microscope for MN. For each sample 1000 cells are scored according to the criteria described [23].

2.3. Cytokinesis-block micronuclei (CBMN) assay

Lymphocyte cultures are set up according to the following standard method [24]. At 44 h, Cytochalasin B (6 μg/ml) is added to the lymphocyte cultures. At the end of incubation time (72 h), cells are harvested by centrifugation and hypotonic solution (0.075 M KCl) is added then left undisturbed for a minute. The cells are transferred into the slides and fixed in Carnoy's fixative (methanol:acetic acid, 3:1) and stained. About 1000 cells are scored from each subject.

2.4. Chromosome aberration assay

0.5 ml whole blood is added to 4.5 ml RPMI 1640 medium and then incubated at 37 °C. At the end of 71 h, 0.0l g/ml colcemid is added to block cells in mitosis. Lymphocytes are harvested at 72 h by centrifuging cell culture medium at 800–1000 rpm for 7 min and adding hypotonic solution (KCl 0.075 M) at 37 °C for 20 min to swell the cells and fixed with methanol and acetic acid (3:1 v/v) fixative. Cell suspension is put onto slides wetted with ice-cold acetic acid (60%) and dried on a hot plate (56 °C for 2 min). 100 complete metaphase cells of the first cell cycle are evaluated under a microscope (100×) to identify numerical and structural CAs according to the International System for Human Cytogenetic Nomenclature ([25,26]).

2.5. Comet assay (SCGE)

Initial DNA damage is determined by alkaline Comet assay, as described [27]. The cover slips are gently removed after placing the slides on ice for 5 min. Slides are immersed in a jar containing a freshly prepared cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10) to which 1% Triton X-100 and 10% DMSO are added just before use. Lysis is done at 4 °C for 1 h in the dark. 5 µl or 10 µl blood samples are mixed with 75 µl or 80 µl of warm low-melting-point agarose (Gibco) at 0.75%, 37 °C, in a microcentrifuge tube and spread over a fully frosted microscopic slide pre-coated with 200 µl of 0.1% agarose by layering a cover slip.

2.6. Statistical analysis

The statistical significance of the differences in the frequencies between groups is calculated. Mean and standard deviation are calculated to assess the difference between the smokers and non-smokers and the level of significance is calculated by an ANOVA. Correlation and Regression analysis are performed between the pack years and chromosomal aberrations of the smoker groups.

3. Results

A total of 160 subjects consist of 80 smokers and 80 non-smokers are analyzed for this study. The uniqueness of the study is the smokers (n = 80) are males having mean ages 40.5 ± 13.35 years, with the majority of smokers being between the ages of 26–35 years. The average number of cigarettes per day is 11.82 ± 4.53 with an average consumption time of 17.12 ± 10.91 years. Pack years an indicator of cumulative smoking dose is estimated at 22.66 ± 17.53 for each and every smoker (Fig. 1). To facilitate further analysis, the smokers are stratified by light (⩽15), medium (16–25) heavy (⩾25) pack year smokers (Table 1). The matching of the non smokers is successfully accomplished on the basis of age as no statistical differences between the two groups are observed.

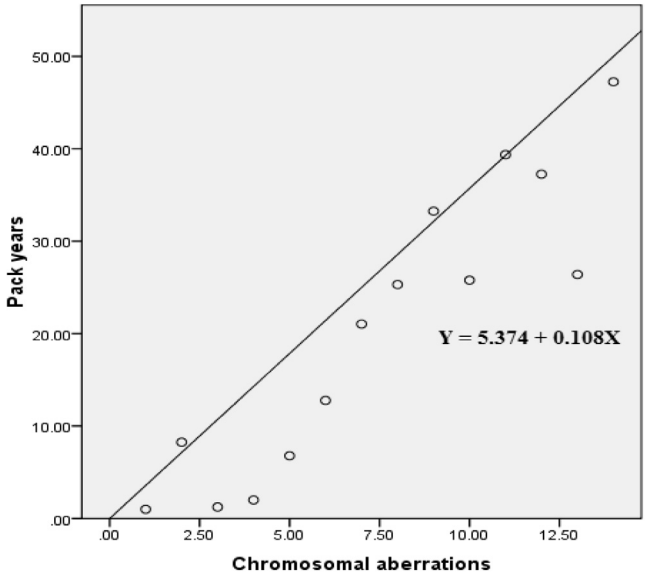
Results of the present study confirmed that the smokers are having higher frequencies of MN in buccal epithelial and blood lymphocytes than non-smokers particularly the heavy smoker group showed significantly increased micronuclei cells. Compared to buccal epithelial cells the blood cells (lymphocytes) showed high results (Fig. 2).

DNA damages in lymphocytes are measured by Comet assay. Nearly 50% increased frequency of damage are observed in the smoker group than non-smoker (Table 2). Among the smoker group, MTL was found high in light smoker; MTM was found high in medium smoker; %TL

Table 1 Characteristics of the study subjects.

Variable	Smokers	Non smokers
Subjects	80	80
Age (years)		
Mean ± S.D.	40.5 ± 13.35	38.66 ± 13.08
Number of cigarettes per day		
Mean ± S.D.	11.82 ± 4.53	NA
Number of packs per day		
Mean ± S.D.	1.17 ± 0.45	NA
Consumption of years		
Mean ± S.D.	17.12 ± 10.91	NA
Pack-years		
Mean ± S.D.	22.66 ± 17.53	NA
Light (⩽15)	35	
Medium (16–25)	13	
Heavy (⩾25)	32	

S.D.: standard deviation; NA: not applicable.
Pack years = number of packs of cigarettes per day × time of consumption in year.



R^2=0.431; Pearson correlation (r) = 0.656; Significant correlation level (99.9%)

Figure 1 Relationship between pack years and total chromosomal aberrations of the smoker group.

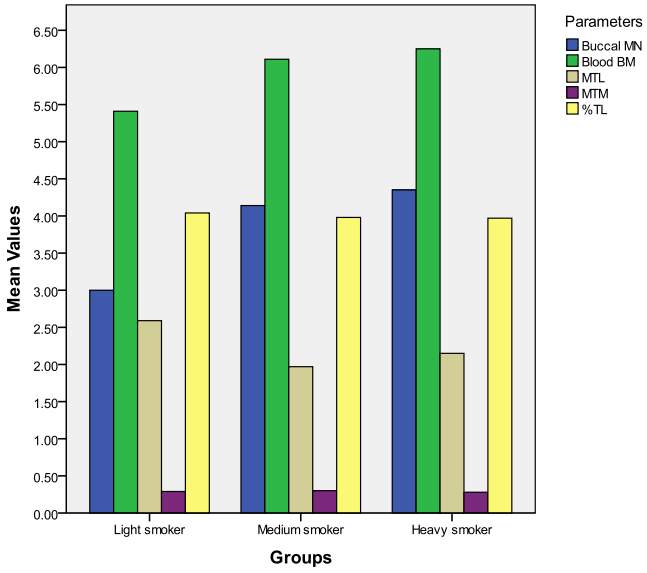


Figure 2 Bar diagram of buccal and blood MN frequencies and Comet assay (MTL, MTM and %TL) of the smoker group.

Table 2 Mean ± S.D. micronuclei and Comet assay study group.

	Non-smokers		Smokers	
	Group I	Group II	Group I	Group II
Micronuclei				
In buccal cells*	0.36 ± 0.52	1.47 ± 0.55	3.46 ± 1.28	4.89 ± 1.61
In blood cells*	0.93 ± 0.86	2.17 ± 0.56	5.82 ± 1.49	6.35 ± 1.57
Comet assay#				
Mean tail length (MTL)	1.10 ± 0.10	1.15 ± 0.11	2.24 ± 0.58	2.07 ± 0.46
Mean Tail Moment (MTM)	0.11 ± 0.07	0.12 ± 0.05	0.3 ± 0.08	0.30 ± 0.07
% Tail length (%TL)	2.13 ± 0.46	2.42 ± 0.57	4.0 ± 0.32	3.98 ± 0.37

Age (mean ± SD): non-smokers Group I-28.80 ± 6.83, Group II-51.82 ± 7.60; smokers Group I-29.58 ± 6.07, Group II-51.97 ± 8.38.
* MN/1000 cells.
# Peripheral blood lymphocytes.

Table 3 Values of MN and Comet assay in smoker groups.

Particulars		Light smokers	Medium smokers	Heavy smokers
Micronuclei*	Buccal	3.0 ± 1.29	4.14 ± 1.62	4.35 ± 1.59
	Blood	5.41 ± 1.70	6.11 ± 1.47	6.25 ± 1.58
Comet assay#	MTL	2.59 ± 0.69	1.97 ± 0.48	2.15 ± 0.48
	MTM	0.29 ± 0.064	0.30 ± 0.09	0.28 ± 0.06
	%TL	4.04 ± 0.44	3.98 ± 0.34	3.97 ± 0.34

* MN/1000 cells.
# Blood lymphocytes.

Table 5 Mean ± S.D. of chromosome aberration for smoker group.

Smokers	CTA	CSA	Total CA
Light	3.16 ± 1.40	1.41 ± 1.03	4.58 ± 2.36
Medium	5.25 ± 1.99	2.44 ± 1.16	7.70 ± 2.50
Heavy	5.90 ± 2.02	3.0 ± 1.21	8.90 ± 2.58

was found high in Light smoker groups but the variations are very little. It is concluded that the smoker group contains nearly similar DNA damage (Table 3).

Based on the cytogenetic analysis chromatid breaks is the most frequent type of Chromosomal aberrations (CAs), with none of the individuals showing CA of the numerical type. The chromosomal aberration frequency of smokers is significantly different from the non-smokers for both chromatid and chromosome type aberrations. The highest CA frequency is observed in heavy smokers compared to light and non smokers as 8.90 ± 2.58, 4.58 ± 2.36 and 4.31 ± 1.17 respectively. Both medium and light smokers showed significantly increased ($P < 0.05$) CA frequencies in comparison to non-smokers (Tables 4 and 5).

4. Discussion

The effect of tobacco smoke is studied in buccal epithelial cells and peripheral blood lymphocytes using Comet assay. The chromosome abnormalities are called as chromosomal aberrations such as change in the number and structure of chromosomes [28]. Tobacco smoking may induce damage to lymphocytes. The inverse relationship found between daily consumption of tobacco and frequency of binucleated (BN) cells may suggest that the genotoxic activity of tobacco smoke is expressed not only as direct damage to DNA in the form of chromosomal aberrations [29].

In cultured PBLs, the formation of CSAs reflected the double-strand breaks mostly generated *in vivo* in G0 stage. For lymphocyte CTAs, double-strand breaks are probably formed from the initial DNA lesions in vitro in S phase. CAs can also be formed spontaneously from double-strand breaks generated by cellular events such as topoisomerase action, DNA replication, V(D)J recombination, transposable elements, and fragile sites, and in excision repair of oxidative DNA damage, apurinic and apyrimidinic sites and deamination products. Formation of double strand breaks in unreplicated DNA in G0 and G1 phases of the cell cycle leads to formation of CSAs, whereas double-strand breaks generated in duplicated DNA in the S and G2 phases give rise to CTAs [30].

The frequencies of CA are found to be higher in the exposed groups and the aberrations predominately observed are of chromatid-type. Smoking is found to have considerable effect on the frequency of CA in exposed subjects. With the Comet assay for DNA damage, a significant increase in comet

Table 4 Frequency (mean ± S.D.) of different chromosome aberrations in smoker and non-smoker subjects.

Chromosome aberrations	Non-smokers		Smokers	
	Group I	Group II	Group I	Group II
Chromatid-type aberration (CTA)	3.17 ± 1.04	4.41 ± 0.94	4.09 ± 1.44	6.48 ± 2.09
Chromosome-type aberration (CSA)	0.45 ± 0.64	0.82 ± 0.61	2.0 ± 1.03	3.15 ± 1.16
Total CA	3.63 ± 1.40	5.23 ± 0.80	6.09 ± 2.28	9.64 ± 2.28

tail frequency is also observed in exposed subjects compared to control [19] which is similar to our findings.

Cigarette smoking is responsible for a substantial number of human health problems [31] and their causal relationship between smoking, the induction of biological effects, and the extent of the disease burden among smokers. In our study, a significantly higher frequency of CA is observed among smokers compared to non-smokers. Our CA data are in agreement with other cytogenetic studies among smokers [32]. Various Studies' results showed an association between MN frequency and cancer risk is inferred from mechanistic similarities with CAs, which are shown to be predictive for cancer ([33–35]).

MN studies reflect the carcinogenic effect of smoking which showed an increased frequency of MN due to tobacco smoke. There is always an increased frequency of MN in heavy smokers. In our studies, Total CAs are significantly increased in the heavy smoker group and their frequency is found higher in PBL than buccal epithelial cells. Similarly the MTL, MTM and %TL frequencies are found high in peripheral blood lymphocytes than buccal epithelial cells of then smoker group. Significantly increased frequencies of CAs are observed in early harvest lymphocyte cultures from groups of smokers compared to non-smokers ([36–38]). There is a significant increase in the buccal and lymphocyte MN frequency of exposed subjects than control [26].

In this study the frequency of micronuclei in cytokinesis-blocked lymphocytes and their total CA are significantly increased in smoker subjects. This study showed a clear convergence between the genetical damages induced by tobacco smoke such as DNA damage i.e. chromosomal damage, micronuclei formation and these are considered as the experimental evidence that tobacco smoke acts as mutagen.

5. Conclusion

The present study is focused about the genetical aberrations in smoking subjects based on their age and pack years by MN and Comet assays. The results confirmed that the frequency of MN is high in buccal epithelial and blood lymphocytes of smokers than non-smokers. Among the three smoker groups, heavy smokers showed significantly increased MN. The mean length of the DNA breakage is increased in smokers. Total CA is found high in Group II smoker group. Exposure of smoke causes severe chromosomal alterations which act as mutagens and pave way for various genetical abnormalities.

Conflict of interest

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