ORIGINAL RESEARCH

Comparative evaluation of genotoxicity in tobacco users versus non tobacco users: a pilot study

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Abstract

Background: Tobacco use continues to be prevalent in our society. It contains many substances which are genotoxic in nature. The genotoxicity of these substances can be studied in human peripheral blood lymphocytes by estimating the average number of micronuclei (MN) by cytokinesis block micronuclei assay (CBMN assay).

Aims and objectives: To find out whether there is a difference in genotoxicity between tobacco users and non tobacco users.

Materials and methods: Five ml of fresh blood was obtained from 15 persons with no habit of tobacco use and 15 persons using tobacco either smoking, chewing or combination of both with clinically normal mucosa in the age group of 20-40 years reporting to the out patient department of PMS College of Dental Sciences & Research, Thiruvanathapuram. Cytokinesis block micronuclei assay was performed in all the samples and frequency of micronuclei was estimated.

Results: Results showed a significant increase in the number of micronuclei among tobacco users when compared to that of non tobacco users.

Conclusion: Estimation of frequency of micronuclei by CBMN assay can be used to assess the genotoxicity present in blood and helps in identifying tobacco users who are at a high risk for development of cancer.

Introduction

The wide spread use of tobacco products worldwide continues to be the greatest threat to global health. In developing

countries, the tobacco companies are targeting the youth and the prevalence is said to be rising among men and women alike. Besides the systemic problems created by tobacco products, it also causes many oral health problems. It can vary from staining of the dentition, gingival diseases and periodontal diseases to major life threatening conditions like oral cancer.[1]

Tobacco is used in many ways, mostly smoked (cigarettes) and smokeless tobacco. Cigarette contains about 80 cancer causing agents which include noxious chemicals like benzaanthracene, furan, N-nitrosodimethylamine, 2-Toluidine, formaldehyde, acetaldehyde, phenol, isoprene, benzene *etc*.[2] Smokeless tobacco comes in two ways, chewing tobacco and snuff. Moist snuff is taken orally while dry snuff is powdered tobacco that is mostly inhaled through the nose. One of the sites of highest risk for cancer due to smoking is the lung, followed by oral cavity and larynx. The risk increases with increase in number of cigarettes smoked.[3]

In the Human Micronucleus (HUMN) project conducted by Stefano Bonassi et al in 2003 to analyse the effect of smoking habit on the frequency of micronuclei in human lymphocytes using cytokinesis block micronuclei(CBMN) assay, a significant increase in micronuclei frequency was observed only in heavy smokers who were not exposed to other genotoxic agents.[6]

Micronuclei ('small nucleus') are cytoplasmic chromatin masses that originate from chromosome fragments or whole chromosomes that fail to engage with the mitotic spindle, lag behind and not carried to the opposite poles during the anaphase of cell division. These chromosome fragments develop nuclear membranes and form a third nucleus in the cytoplasm and are referred to as micronuclei.[7] Quantification of frequency of micronuclei in the lymphocytes of peripheral blood using cytokinesis –block micronucleus assay is one of the standard cytogenetic tests for measuring chromosomal damage because of its good reproducibility and reliability.[7,8] The assessment of increased frequency of micronuclei helps in identification of high risk group of individuals susceptible to cancer.[8,9]This would further help in implementation of tobacco cessation programmes among high –risk individuals for cancer.

The Human Micronucleus (HUMN) project was conducted by Stefano Bonassi et al in 2003 to analyse the effect of smoking habit on the frequency of micronuclei in human lymphocytes using cytokinesis block micronuclei(CBMN) assay. The study was done by re- analysing the pooled data of HUMN database (24 databases from 16 countries). In their analysis, only weak associations were obtained between cigarette smoking and micronuclei frequency. But in most of these studies, tobacco use overlapped with old age, alcohol consumption, chronic infections, confirmed systemic illness, history of malignancy and exposure to cytotoxic drugs, chemicals or ionising radiation. A significant increase in micronuclei frequency was observed only in heavy smokers who were not exposed to other genotoxic agents.[6]

In our study we have ruled out all the possible variables that can affect the frequency of micronuclei and restricted our study to tobacco and non tobacco users aged between 20 and 40 years with no visible oral changes. CBMN assay was conducted with the objective of assessing the difference in micronuclei frequencies in the peripheral blood of the study subjects with that of the control subjects. By this, genetic damage caused by tobacco alone can be identified at a very early stage, even before the appearance of clinical signs of cancer. This would further help in implementation of tobacco cessation programmes among high –risk individuals for cancer.

Materials and methods

After obtaining institutional ethical committee clearance, the study was conducted in patients reporting to the outpatient department, PMS College of Dental Sciences & Research, Thiruvananthapuram. The laboratory procedures was carried out in '*Genitika*' (an ISO ceritified laboratory), Thiruvananthapuram. Control group consisted of 15 persons with no habit of tobacco use (smoking as well as tobacco chewing) and having clinically normal oral mucosa and study group consisted of 15 persons using tobacco either smoking, chewing or combination of both. (Current smokers – CDC classification).

The criteria for selecting the tobacco smokers, was based on the definitions given by US center for disease control and prevention (CDC).

- Never Smokers – Adults who have never smoked a cigarette or who smoked fewer than 100 cigarettes in their entire lifetime.
- Former Smokers – Adults who have smoked at least 100 cigarettes in their lifetime, but say they currently do not smoke.
- Nonsmokers – Adults who currently do not smoke cigarettes, including both former smokers and never smokers.
- Current Smokers – Adults who have smoked 100 cigarettes in their lifetime and currently smoke cigarettes every day (daily) or some days (nondaily).[10]

Patients above 20 years and below 40 years, patients with clinically detectable changes in the oral mucosa, patients with alcoholism, confirmed systemic illness or with history of malignancy, patients who reported of infections within 3 months of study and who were exposed to cytotoxic drugs, chemicals, or radiation therapy were excluded from the study.

The number of micro nuclei was considered as the dependent variable while age, sex and disease status were the independent variables.

For the CBMN assay, 5 ml of fresh blood was taken after obtaining written informed consent from each subject by venipuncture and transferred to heparinised vacutainers

and lymphocytes isolated using lymphoprep. The lymphocytes were then suspended in Roswell Park Memorial Institute1640 medium and centrifuged for 10 minutes. The supernatant was removed and this step was repeated three or four times. The lymphocytes were cultured using RPMI 1640 medium containing 15% foetal calf serum and phytohaemagglutinin for 72 hours at 37^{0}C. After 44 hours, cytochalasin-B was added. After 28 hours, the whole contents were centrifuged for 10 minutes, the supernatant removed & the pellet shaken in a cyclomixer. Ten ml of 0.075 M KCL was added to the cell button for 10 minutes. Two drops of fixative was added and centrifuged for 10 minutes. The supernatant was removed and the cell button mixed in a cyclomixer. Ten ml of fixative was added and centrifuged for 10 minutes and repeated until the cell button became white. Cell suspension was dropped onto slides, fixed, stained with Giemsa stain and examined at 100X magnification. The number of micronuclei in no less than 1000 binucleated cells was scored.

The criteria for identification of micronuclei as given by Heddle and Countryman (1976) was followed for the present study and are stated below.

1. Diameter less than 1/3rd the main nucleus.
2. Non refractility (to exclude small stain particles).
3. Colour same or lighter than the nucleus.
4. Location within three or four nuclear diameters of a nucleus; and not touching the nucleus.
5. No more than two micronuclei associated with one nucleus.[7]

Results

Since the count of micronuclei is a discrete variable, the analysis was done using non parametric method namely Mann-Whitney U test and descriptive measure was the median number of micronuclei counts. There was a significant difference in the frequency of micronuclei among the control group and study group.

Table 1. Distribution according to micronuclei frequency of non tobacco users

MICRONUCLEI FREQUENCY	NUMBER OF SUBJECTS
9.64	1
10.04	1
10.40	2
10.44	1
10.50	1
10.60	1
10.70	1
10.80	1
11.00	3

11.20	3

Table 2. Distribution according to micronuclei frequency of tobacco users

MICRONUCLEI FREQUENCY	NUMBER OF SUBJECTS
12.00	1
12.80	1
12.82	1
12.87	1
13.06	1
13.20	1
13.40	1
13.41	2
13.46	1
13.64	1
13.80	1
13.88	1
14.14	1
14.18	1

Table 3. Comparison of micronuclei values of tobacco users and non tobacco users

STATUS	MEAN	MEDIAN	STANDARD DEVIATION (SD)
Non tobacco users (controls)	10.67	10.70	0.45
Tobacco users (study group)	13.34	13.41	0.58

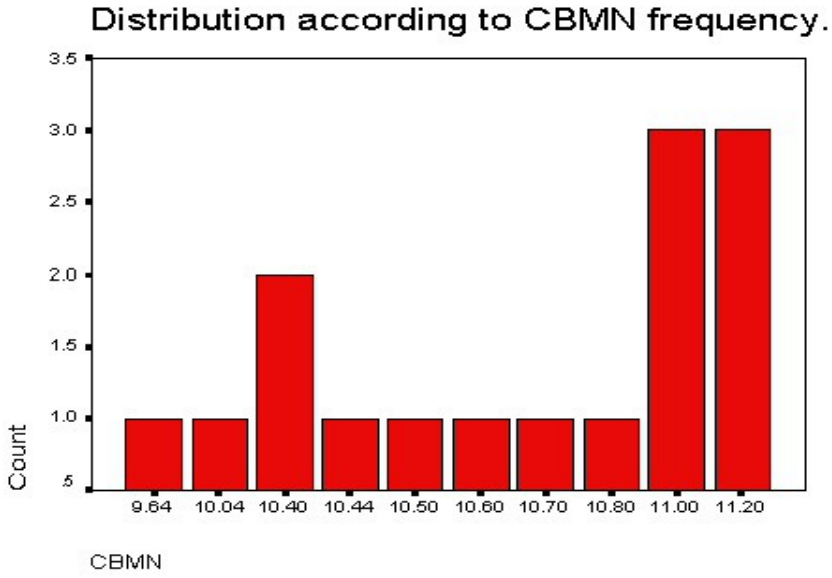

Figure 1. Micronuclei frequency in non tobacco users

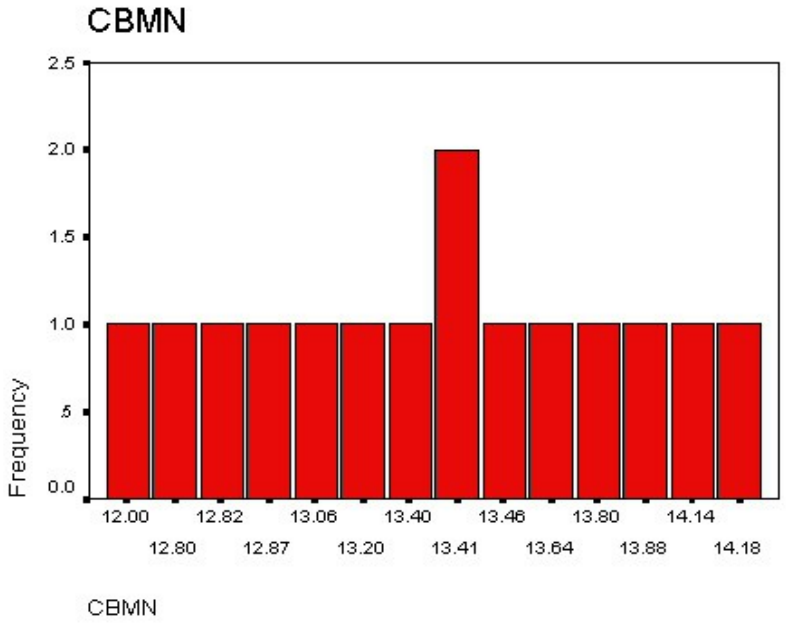

Figure 2. Micronuclei frequency in tobacco users

Mann- Whitney U test value= .000; p value = .0001; there is highly significant difference in the median micronuclei counts between smokers and non smokers.

Discussion

The usage of tobacco is prevalent throughout the world. Tobacco is widely used in different forms, especially smoked and smokeless forms. Cigarettes, the smoked form of tobacco is said to be used by over 1.1 billion people. In developing countries, smokeless tobacco is also extensively used, mainly in South- East Asian countries. Tobacco has been long established as a multisite carcinogen in humans and said to be responsible for approximately 30% of all cancer deaths in developed countries.

Tobacco users have markedly increased risks of lung cancer and oral cancer. The risk of oral squamous cell carcinoma has increased in the recent past around the world due to increased use of tobacco products and is a major cause of morbidity and mortality worldwide. A fundamental factor in the poor prognosis of oral squamous cell carcinoma is that they are diagnosed in advanced stages and thus treated very late. Treatment is often surgery, and in advanced cases, surgery is followed by radiation therapy. The cost of effective treatment and further rehabilitation is very high, with most cases resulting in relapse and eventually death. Most of this can be averted if the susceptible individuals can be identified at an early stage even before the appearance of clinical symptoms. Here comes the role of chromosomal biomarkers. Studies have shown that chromosome damage is an early indicator of carcinogenesis and this can be used as a tool for identifying high risk patients. Many biomarkers have been identified and among them, micronuclei is said to be the most reliable biomarker for genotoxicity. The micronuclei arise as a result of genomic damage to cells and are seen in the cytoplasm in the anaphase stage of the cell cycle. The CBMN assay in peripheral blood lymphocytes has been developed as a precise method for the assessment for micronuclei. Baseline MN frequencies in cultured human lymphocytes provide an index of accumulated genetic damage. Different forms of tobacco are associated with increased number of MN which is indicative of its genotoxic ability and thus its ability to cause cancer.

Our study among 30 individuals revealed that there is a significant increase in frequency of micronuclei among tobacco users when compared with non- tobacco users thus confirming the genotoxicity of substances contained within tobacco products.

Cruz *et al* in their study on human micronuclei counts using CBMN assay concluded that increase in micronuclei frequencies were most strongly correlated with the dose of ionising radiation, but age, alcohol consumption and smoking habits also affected micronuclei frequencies.[11] In a similar study on radiological workers, Thierens *et al* observed no correlation between micronuclei frequency and smoking habits. [12]

Different methods other than CBMN assay has been employed in assessing the frequency of micronuclei. One such method is the chromosomal aberrations assay. Alsatari *et al* assessed DNA damage in lymphocytes of water pipe smokers using chromosomal aberrations assay and found that tobacco smoking using cigarette or water pipe induces significant aberrations in

lymphocytes and that the level of chromosomal aberrations was higher in water pipe smokers than cigarette smokers.[13]

Assessment of micronuclei from exfoliated cells of oral cavity has been also used to detect chromosome damage by epithelial carcinogens like tobacco smoke. Mohanta *et al* analysed the genotoxicity of tobacco and alcohol on oral mucosal cells of 136 patients suffering from precancerous lesions and oral squamous cell carcinoma and observed that the frequency of micronuclei was significantly higher in patients who used tobacco and alcohol when compared to controls.[1]

The results from the present study shows that micronuclei may possibly be used as a predictive biomarker of cancer risk and that this test may be useful in identifying patients with more genetic damage which in turn will help in motivating the patient for cessation of the habit.

Conclusion

Tobacco has known carcinogenic potential because of the many genotoxic substances present in it which ultimately leads to development of cancer in individuals. The results obtained from this pilot study, shed light on the fact that the genotoxicity in tobacco users is significantly higher when compared to that of people who do not use tobacco. This would inturn help in implementation of tobacco cessation programmes among high –risk individuals for cancer.A larger sample size with longer follow up is required to further substantiate the relationship between increase in micronuclei in peripheral lymphocytes and risk of cancer development in individuals.

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