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The Effects of Cigarette Smoking on Micronucleus Frequencies in the Peripheral Blood Lymphocytes & Buccal Epithelial Cells of Smokers in Bankura Disrtict, West Bengal

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Abstract

The genotoxic damage that Tobacco smoke produces was evaluated using the micronucleus assay from the peripheral blood lymphocyte & Buccal epithelial cells of smokers in Bankura district, West Bengal. About 45 years before Schmid and Maier (1976), Schmid(1976) convincingly demonstrated in mouse that MN test is one of the reliable bioassays to detect the genotoxic potential of environmental agents. Several testing protocols for detecting the mutagenic potentials of odd agents are in use, of which Micronucleus test (MNT) has been claimed to be an acceptable short term screening method. MN are small chromatin containing bodies arising from chromosome fragments that were not incorporated into daughter nuclei following mitosis. MN form only in dividing cells in the case of erythropoiesis in erythroblasts. The newly formed erythrocytes which are formed by extrusion of nuclei from erythrocytes still contain rRNA and are called polychromatic erythrocytes (PCEs), only the PCEs will gave micronuclei. We concluded that the higher frequency of micronuclei directly associated with the decrease of efficiency of DNA repair and increase of genomic instability. Micronucleus assay in the buccal cell is sensitive, practical, inexpensive method for monitoring genetic damage in human. Further researches are needed not only for count micronuclei but also to capture the frequency of apoptosis and necrosis cells to detect the further evaluation of cellular and tissue level biological damage.

Keywords: Buccal Cells, Micronucleus Assay, Genotoxicity, Smoking

Introduction

Tobacco is reported to contain more than 60 genotoxic chemicals and radionuclide's, which promote various types of cancers and cardiovascular diseases, myocardial infarction, cerebral vascular diseases, chronic obstructive pulmonary disease and emphysema (Levitz et al., 2004). The consumption of tobacco products either for smoking or for chewing is found to be associated with oral tissue neoplasia. Over 80 % oral cancer patients are tobacco users (Johnson et al., 2001). Among other carcinogenic chemicals present in tobacco, tar plays important role to increase the risk of diseases. The risk is enhancing in the developing nations because the cigarettes sold in these countries, usually contain higher tar content leading to a potential increase in tobacco related diseases in these regions (Nichter and Cartwrigh, 1991). The United States Centre for Disease Control and Prevention (US CDCP) described tobacco use as a single most important preventable risk to human health and an important cause for premature death worldwide (US CDCP, 2010).For the early detection of tobacco induced diseases, certain biomarkers are identified by the researchers. The frequency of chromosomal aberration and micronuclei (MN) frequency assay are considered as effective biomarkers. One of the best techniques for studying the effects of cigarette smoking, on the genetic stability in human cells is the micronucleus assay (Nersesyan et al., 2006). Micronucleus is an oval cytoplasmic chromatin mass in the extra nuclear vicinity. It originates from abnormal mitosis. It consists of eccentric chromosome or chromatic fragments or whole

chromosomes which failed to reach spindle poles during mitosis. The frequency of micronucleus has been used as a biomarker for assessment of DNA damages. Therefore, the frequency of micronuclei observed in blood cells is considered as an appropriate index to monitor the genotoxic effect of cigarette smoking. Stich et al. (1982) first introduced the micronucleus assay in buccal epithelial cells. The number of micronucleus is related to increasing degree of impact of carcinogens. Another advantage of micronucleus assay is that the formation of micronuclei happened before the appearance of clinical symptoms of cancer. Buccal cell changes are associated with tobacco use. When compared with other body sites, the mouth offers a unique opportunity for defining biomarkers because the mouth permits non invasive, repetitive longitudinal studies of tobacco associated acute and chronic diseases (Proia et al., 2006). In the present study, micronucleus assay was carried out in peripheral blood Lymphocyte and buccal epithelial cells in order to evaluate genotoxic impact of cigarette smoking in smokers of Bankura District, West Bengal.

Objective of the Study

Many physical and chemical factors affect stability of our genome and can contribute to the development of civilization diseases, such as cancer, cardiovascular disease, chronic obstructive pulmonary disease or neurodegenerative diseases. Tobacco is one of the most harmful mutagenic chemical. It contains many mutagenic and carcinogenic agents. Genotoxicity testing allows for an assessment of their impact on humans and biota. Nearly 20 types of well-described in vitro or in vivo genotoxicity tests are presently used, of which MN test has been claimed to be an acceptable short term sereening method. MN is a small; chromatin containing round-shaped body visible in the cytoplasm of cells.MN is considered to be caused by DNA damage or genomic instability. MN can occur as a result of natural processes, such as metabolism or aging or can be induced by many environmental factors, hazardous habits and different diseases. Globally large number of people dies every year due to tobacco smoking. Both men and women face increased risk of incurable and fatal diseases. In the rural areas of West Bengal both men and women have the habit of tobacco smoking. Considering above the present work micronucleus assay was carried out in peripheral blood Lymphocyte and buccal epithelial cells in order to evaluate genotoxic impact of cigarette smoking in smokers of Bankura District, West Bengal has been undertaken.

Review of Literature

MN are small, extranuclear bodies additional to the main nucleus in a cell. They are formed when whole chromatids, chromosomes and acentric fragments are left behind and finally excluded from the daughter nuclei at mitosis (Heddle et al. 1983; Ford et al. 1988; Lindholm et al. 1991; Ford and Correll 1992). The MN assay was used for the first time in vitro in radiation experiments with roots of Vicia faba in 1959 (Evans et al. 1959). Laggards cannot move towards the poles, because they are detached from the mitotic spindle or, alternatively, have bipolar kinetochore orientation, as described by Cimini et al. (2001). Besides these fundamental mechanisms, some MN may have their origin in fragments derived from broken anaphase bridges (Cornforth and Goodwin 1991; Saunders et al. 2000; Huang et al. 2011) formed due to chromosome rearrangements (Norppa and Falck 2003). In the microscope, a micronucleus is visualized as an extra small nucleus beside the main nucleus of an interphase cell. In addition, MN can be assessed much more easily, quickly, and objectively .It is possible to score thousands instead of hundreds of cells per treatment or subject, which leads to a greater accuracy of the test. Nowadays it is possible to use automated methods for micronucleus analysis (Decordier et al. 2011; Rossnerova et al. 2011). Furthermore, structural and numerical chromosome mutations are concerned in many human genetic diseases and are involved in the initiation or progression of cancer (Kirsch-Volders et al. 2000). On the other hand, since cells need to survive at least one nuclear division for micronucleus formation, some damaged cells may not be detected because they have been lost or unable to divide (Bonassi and Au 2002). In the early seventies, Schmid (1975) used micronucleus analysis in bone marrow erythrocytes of mice as a technique to study the genotoxicity of various exposures in vivo. Since 1983, the erythrocyte micronucleus test (MN test) has been included in OECD test guidelines and is the most frequently used in vivo assay for routine genotoxicity screening of chemicals (OECD 1997). Kirsch-Volders, M.; Fenech, M.; Bolognesi, C. (2018) Works on Validity of the Lymphocyte Cytokinesis-Block Micronucleus Assay (L-CBMN) as biomarker for human exposure to chemicals with different modes of action. Franciesa, F.Z.;Wainwrightc, R.; Pooled, J.; de Leeneere, K.; Coenee, I.;Wiemee, G.; Poirelg, H.A.; Brichardh, B.; Vermeuleni, S.; Vral, A.; et al.(2018) works on " Diagnosis of Fanconi Anaemia by ionising radiation- or mitomycin C—Induced micronuclei DNA Repair". Rodrigues, M.A.; Beaton-Green, L.A.; Wilkins, R.C.; Fenech, M.F. (2018) works on "The potential for complete automated scoring of the cytokinesis block micronucleus cytome assay using imaging flow cytometry". Wang, Q.; Rodrigues, M.A.; Repin, M.; Pampou, S.; Beaton-Green, L.A.; Perrier, J.; Garty, G.; Brenner, D.J.; Turner, H.C.; Wilkins, R.C. (2019) works on " Automated Triage Radiation Biodosimetry: Integrating Imaging Flow Cytometry with High-Throughput Robotics to Perform the Cytokinesis-Block Micronucleus Assay". Ye, C.J.; Sharpe, Z.; Alemara, S.; Mackenzie, S.; Liu, G.; Abdallah, B.; Horne, S.; Regan, S.; Heng, H.H. (2019) works onMicronuclei and Genome Chaos: Changing the System Inheritance. Genes. Bolognesi, C.; Fenech, M.(2019) works on "Micronucleus Cytome Assays in Human Lymphocytes and Buccal Cells". Aguiar Torres, L.; Dos Santos Rodrigues, A.; Linhares, D.; Camarinho, R.; Nunes Páscoa Soares Rego, Z.M.; Ventura Garcia, P. (2019)works on Buccal epithelial cell micronuclei: Sensitive, non-invasive biomarkers of occupational exposure to low doses of ionizing radiation.

Concepts and Hypothesis

MN are formed from both whole and fragmented chromosomes that lag behind in cell division and are eventually left outside the daughter nuclei (Norppa and Falck 2003). In general, micronucleus analysis is utilized in both genotoxicity testing and biomonitoring of genotoxic exposure and effect in humans. MN are particularly considered a useful biomarker of genotoxic effects. In principle, the micronucleus assay (MN assay)allows the detection of both aneugenic agents and clastogenic agents and can be applied, in addition to peripheral lymphocytes, to cells exfoliated from buccal, nasal and urothelial mucosa (Albertini et al. 2000; Pastor et al. 2001; Norppa and Falck 2003; Holland et al. 2008). The analysis of MN has been used to quantify exposure to genotoxic chemicals and radiation in a large number of studies. MN can be observed in almost any cell type, and for this reason many variations of the assay exist. Micronucleus analysis is employed in studies with humans and laboratory animals following in vivo or in vitro exposures (Tucker and Preston 1996). One could say that the use of MN as an indicator of (Kirsch-Volders et al. 2000) chromosomal damage has become a standard assay in both genetic toxicology and human bio monitoring studies. Micronucleus assay is considered to be one of the widely used short term procedures for the evaluation of exposure to mutagens and carcinogens. In the present study, the genotoxic effect of smoking was evaluated thoroughly. Micronucleus assay was carried out in peripheral blood Lymphocyte and buccal epithelial cells.

Considering above the present work micronucleus assay was carried out in peripheral blood Lymphocyte and buccal epithelial cells in order to evaluate genotoxic impact of cigarette smoking in smokers of Bankura District, West Bengal has been undertaken.

Research Design

Materials and Methods

Peripheral blood samples collected from smoker and non-smoker group were used for the assessment of micronuclei assay.

Micronuclei Assay in Human Peripheral Blood Lymphocyte

For the micronuclei assay study, human peripheral blood Lymphocyte culture was carried out following the method of Fenech and Morley (1986). 5 ml of peripheral blood from each smoker and non-smoker person was collected using heparinised syringe for peripheral blood Lymphocyte culture. The micronuclei preparations were processed and stained with Giemsa. About 2.0 ml of venous blood each from the experimental (smokers) and control (Non smokers) subject was drawn into a sterile heparinized syringe and 0.5 ml of the blood (about 30 drops) was inoculated under aseptic conditions into a culture vial containing 5.0 ml of culture medium, 1.0 ml of AB serum and 0.2 ml of PHA. The cultures were incubated at 37°C for a period of 72 h and were shaken periodically twice a day in order to facilitate proper mixing of the medium and cells in culture. A test slide was prepared by placing a drop of the cell suspension on a clean chilled slide and dried immediately at 40°C for a few seconds on a hot plate. The slide was examined under a microscope to see whether the concentration of cells and the spread of the chromosomes enabled detailed examination of metaphases. The rest of the slides were prepared after making suitable dilutions of the cell suspension with fresh fixative.One thousand well spread metaphase plates of each group were screened under oil immersion 100x lens and observed under microscope and selected metaphases were photographed, for observing micronuclei frequency.The significance between means of observed frequency of micronuclei in smokers and non- smokers was tested by applying ANOVA. The level of significance was set at $P < 0.05$.

Micronuclei Analysis in Buccal Epithelial Cells

Before starting the buccal cell collection, the persons were requested to rinse the mouth thoroughly with water in order to remove cell debris and food particles. Samples were collected using small headed tooth brush (one for each person). The tooth brush was gently but firmly rotated 10 times against the cheek wall in circular motion to collect the buccal cells. The buccal cells collected from each cheek were transferred to a 30 ml container each containing 20 ml of sarcomono fixative (Thomas et al., 2009). The buccal cell suspension fixed in the sarcomono solution was stored at 4ºC.The cell suspension from the two containers were transferred into two Centrifuge tubes and centrifuged for 10 minutes at 580 X g. The supernatant was aspirated leaving approximately 2ml of cell suspensions. The cells are resuspended by adding 8ml of buccal cell buffer and centrifuged for 10 minutes at 580 X g. The supernatant was aspirated leaving approximately 1ml of cell suspension and 10 ml of buccal cell buffer was added the cell suspension was centrifuge for 10 minutes at 580 X g. The supernatant was aspirated leaving 1ml of cell suspension and 5ml of buccal cell buffer was added. The cell suspension was homogenized for 2 to 3 minutes using a hand held tissue homogenizer. Air dried with 10 minutes at room temperature, the slides were stained with 5 % Giemsa stain for 10 or 20 minutes. After the staining, the slides were rinsed in running water for 5 minutes and air dried in room temperature. The slides were evaluated for micronuclei frequency under the microscope at 100x magnification.Above 1000 binuclear cells (BN cells) were scored for the presence of micronuclei. The significance between means of observed frequency of micronuclei in smokers and non smokers was tested by applying ANOVA. The level of significance was set at $P < 0.05$.

Results

Aggregated information about age, duration and intensity of smoking as well as results of conducted cytogenetic analysis in smokers and non-smokers are presented in table-2. Equal number of smokers (80) and Non smokers (80) were selected for the present study. All of them were only males. The sampled smokers and non-smokers are divided each into five age groups namely group I : 18-28 years ;

group II : 29 -38 years; group III : 39 -48 years; group IV : 49 -58 years ; group V : above 59 years .

Micronuclei Frequency in Peripheral Blood Lymphocyte

The sample size of the five age groups among non smokers ranged from 11 to 25 and among smokers ranged from 11-22. The total number of observed cells was, 1000 for each sample and a total of 80,000 cells were scored for each cohort (smokers and non-smokers). The frequency of micronuclei (MN) in peripheral blood Lymphocyte of non-smoker is presented in Table 1. It ranged from 0.38 ± 0.48 to 3.09 ± 1.67. The average micronuclei frequency in non-smoker was 1.79 ± 0.89 MN/1000 BN cell. The frequency also increases with age of the smokers and non-smokers. Among non-smokers ranged from 0.38 in the age group 18-28 years to 3.09 in age group above 59 years and smokers from 1.81 in age group 18-28 years to 3.9 in age group above 59 years.

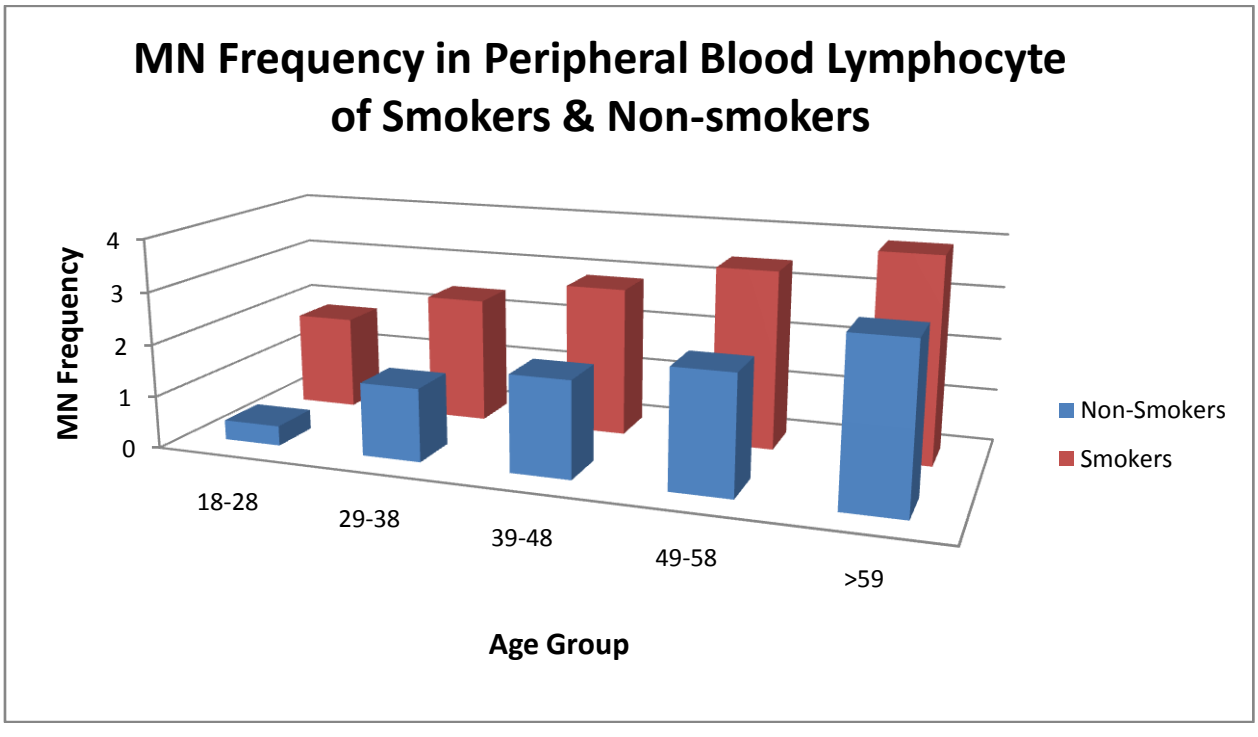
Table 1 Micronuclei Frequency in Peripheral Blood Lymphocyte of Non – Smokers

Age Group	Age Year	No. of Samples	No. Of observed cells	MN Frequency
Group I	18-28	13	1000	0.38 ± 0.48
Group II	29-38	15	1000	1.4 ± 0.87
Group III	39-48	25	1000	1.84 ± 0.73
Group IV	49-58	16	1000	2.25 ± 1.8
Group V	>59	11	1000	3.09 ± 1.67
Range				0.38-3.09
Mean ± SD				1.79 ± 0.89

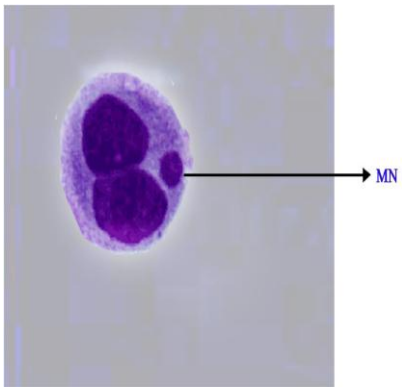
Table -2 , presents the data on frequency of micronuclei in peripheral blood Lymphocyte of smokers which ranged from 1.81 ± 0.93 to 3.91 ± 1.44. The overall mean value was 2.88 ± 0.73. The ratio between the mean frequency of micronuclei among smokers and non-smoker age groups is also presented in Table-2. It ranged from 1.2 – 4.7 with a mean ratio value of 2.12±1.29, indicating that the frequency of micronuclei among smokers is distinctly higher than in nonsmokers.

Table 2 Micronuclei Frequency in Peripheral Blood Lymphocyte of Smokers

Age Group	Age Year	No. of Samples	No. Of observed cells	MN Frequency	Smokers/Non-smokers Ratio
Group I	18-28	13	1000	1.81 ± 0.93	4.7
Group II	29-38	15	1000	2.42 ± 1.29	1.7
Group III	39-48	25	1000	2.86 ± 1.63	1.5
Group IV	49-58	16	1000	3.42 ± 1.25	1.5
Group V	>59	11	1000	3.91 ± 1.44	1.2
Range				1.81-3.91	1.2-4.7
Mean ± SD				2.88 ± 0.73	2.12 ± 1.29



Micronuclei Frequency in Peripheral Blood Lymphocyte in Different Age Group of Smokers and Non Smokers



Micronucleus Observed in Peripheral Blood Lymphocyte of Smoker

Micronuclei Frequency in Buccal Epithelial Cells

The frequency of exfoliated buccal epithelial micronuclei observed among various age groups of non-smokers is presented in Table -3. The frequency of micronuclei in non-smokers ranged from 0.15 ± 0.36 to 2.09 ± 1.62. The average micronuclei frequency was 1.06 ± 0.70. The frequency also increased with age of the smokers and non-smokers. Among non-smokers the frequency ranged from 0.15 in the age group 18-28 years to 2.09 in age group above 59 years and in smokers from 0.72 in age group 18-28 years to 3.08 in age group above 59 year.

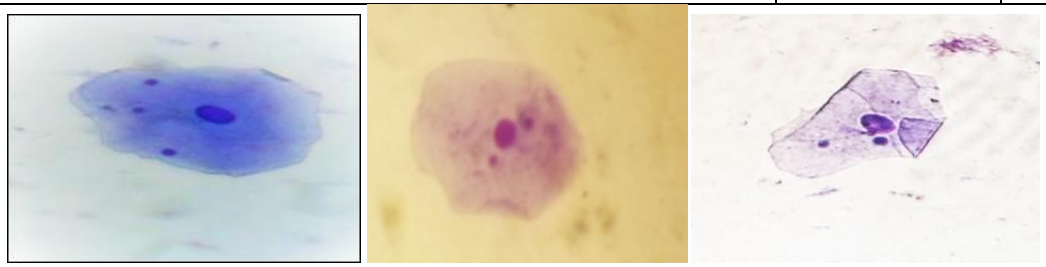
Table: 3. Micronuclei Frequency in Buccal Epithelial Cells of Non- Smokers

Age Group	Age Year	No. of Samples	No. Of observed cells	MN Frequency
Group I	18-28	13	1000	0.15 ± 0.36
Group II	29-38	15	1000	0.46 ± 0.49
Group III	39-48	25	1000	1.04 ± 0.82
Group IV	49-58	16	1000	1.56 ± 1.27
Group V	>59	11	1000	2.09 ± 1.62
Range				0.15-2.09
Mean ± SD				1.06 ± 0.70

Table 4 revealed that frequency of micronuclei in smokers ranges from 0.72 ± 0.74 to 3.08 ± 0.86. The overall mean frequency in smoker cohort was 1.86 ± 0.81. The ratio between the mean frequency of micronuclei among smokers and non-smoker age groups is also presented in Table -4.It ranges from 1.4 to 4.8 with a mean ratio value of 2.42 ± 1.28, indicating that the frequency of micronuclei among smokers is distinctly higher than in non-smokers.

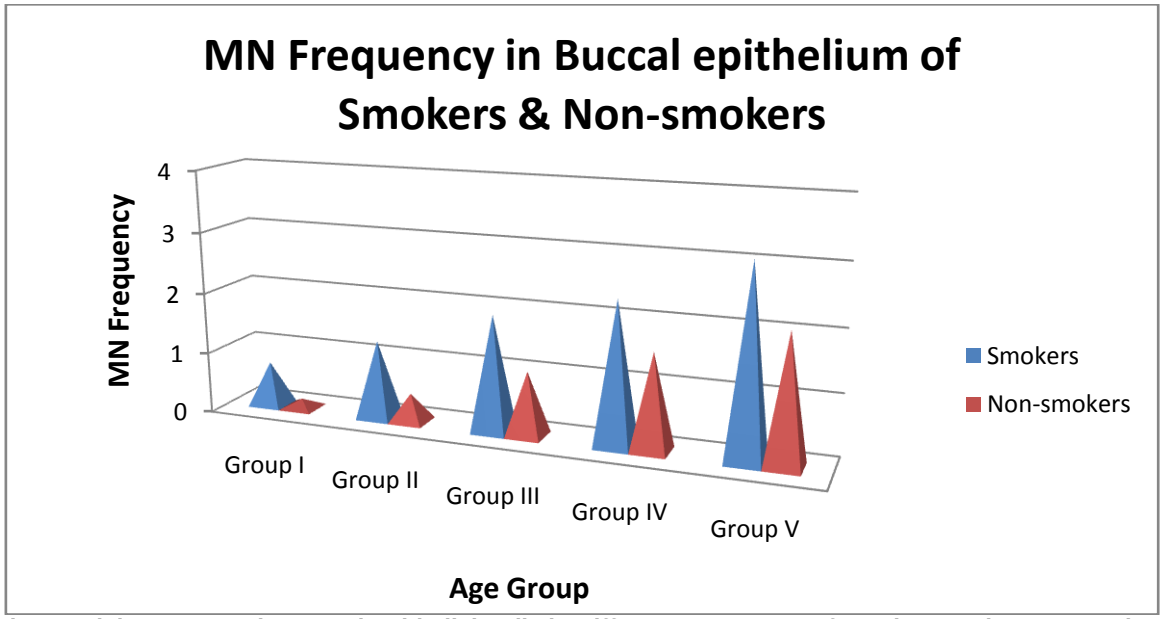
Table 4 Micronuclei Frequency in Buccal Epithelial Cells of Smokers

Age Group	Age Year	No. of Samples	No. Of observed cells	MN Frequency	Smokers/Non-smokers Ratio
Group I	18-28	11	1000	0.72 ± 0.74	4.8
Group II	29-38	14	1000	1.28 ± 0.88	2.7
Group III	39-48	22	1000	1.90 ± 1.20	1.8
Group IV	49-58	21	1000	2.33 ± 0.89	1.4
Group V	>59	12	1000	3.08 ± 0.86	1.4
				0.72 - 3.08	1.4-4.8
				1.86 ± 0.81	2.42 ± 1.28



A **B** **C**

Photomicrographs of exfoliated buccal cells stained using Giemsa stain in micronucleus assay, A: cell with three micro nuclei ; B,C cells with two micronuclei .



Micronuclei Frequency in Buccal Epithelial Cells in Different Age Group of Smokers and Non – Smokers

Conclusion

The World Health Organization (WHO, 2001) reported that the tobacco use has been the number one factor in increasing the risk of various types of cancers like lung, urinary bladder, kidney, pancreas, mouth, throat and stomach cancers. Smoking is also supposed to cause acute leukemia as well as liver and uterine cervix cancer (Wang and Samet, 1987). Shopland (1995) reported that in the US about 38 % of all cancer deaths in males and 23 % of all cancer deaths in females are due to cigarette smoking. The carcinogenicity of cigarette smoke is largely determined by genotoxicants present in cigarette smokes. Cigarette smoke contains more than 4800 chemical compounds and 69 of them are identified as carcinogens (Hoffmann and Hoffmann 1997; Hoffmann et al., 2001; Hecht, 1999). The genotoxic effect of cigarette smoking resulted in genetic aberrations including gene mutation, chromosomal aberrations, micronuclei, sister chromatid exchange, DNA strand breaks etc. (Demarini, 2004). The results of the present study revealed that the frequency of micronuclei significantly increased in smoker cohort as compared to non- smoker cohort ($P < 0.05$). The mean frequency of micronuclei recorded in smokers was 60 % higher (2.9) than in non-smoker (1.8). It was also interesting to note that the micronuclei frequency enhanced with age group of both non-smoker and smoker cohorts. This observation can be attributed to influence of duration of smoking i.e. older people were exposed to cigarette smoke for longer duration than in young smokers. Several studies have evaluated the effects of smoking on the frequency of micronuclei in human peripheral Lymphocyte and increase in the rate of micronuclei formation in smokers has been determined (Au et al., 1991; Tomanin et al., 1991; Haveric et al., 2010). However, some authors have reported that smokers have lower frequency of micronuclei (Landi and Barale, 1999). This reverse condition may be related to activation of cell protective responses that reduce the frequency of micronuclei by inducing resistance towards further, DNA damages in smokers (Gourabi and Mozdarani, 1998). In India, Chandrasekar et al. (2011) investigated the influence of tobacco smoke on cytogenetic parameters such as the frequency of micronuclei and chromosomal aberrations and influence of XRCC1 gene variants on these cytogenetic parameters. The study also observed an enhanced frequency of micronuclei in peripheral blood Lymphocytes and buccal epithelial cells of smokers of active and passive smokers, when compared to that of non-smokers. The mean frequency of micronuclei (2.9 ± 0.7) among smokers of Bankura District was distinctly higher when compared to the mean micronuclei frequency reported for smokers is (1.0 to 1.5. However, Haveric et al. (2010) reported a higher micronuclei frequency in the Lymphocytes of smokers in Bosnia. The elevated micronuclei frequency may be due to intensity of smoking and increased concentrations of genotoxic chemicals present in the tobacco tested in Bosnia. The age and sex of the subjects are being reported as the most important demographic variables affecting micronuclei frequencies (Fenech, 1998). Significantly increased micronuclei frequency in females, registered both in smoker and non-smoker females, may be related to the possible loss of X- chromosome (Fenech et al., 1994). However, Gonsebatt et al. (1997) reported increased micronuclei frequency in male cohort. Study on micronuclei frequency in buccal epithelial cells has an important advantage that neither in vitro cultivation non cell division is required nor assay can be carried out within a short time. In smokers, buccal epithelial cells lining the buccal cavity are periodically exposed to tobacco smoke chemicals (Campain, 2004). Further 90 % of human cancers originate from epithelial cells. Therefore, the study of micronuclei frequency in buccal epithelial cells assumes importance in assessing the genotoxic impact induced by carcinogens, entering the body via inhalation or ingestion (Holland et al., 2008).

The present study also reports that micronuclei frequency buccal epithelial cells of smokers were 58 % higher (1.9) than that of non smokers (1.1). It was also observed that older age group of above 59 years registered higher frequency of micronuclei (3.1) when compared to younger age group of 18-28 years (0.72). The results of the present study was comparable to that reported by many authors (Chandrasekar et al., 2011; Chandrasekar et al., 2014; Suhas et al., 2004; Nefic et al., 2013). However, higher micronuclei frequency ranged from 6.0 to 18.0 in India was also reported (Driceiker et al., 2016 in Turkey; Biswas et al., 2014 in India; Motgi et al., 2014 in India ; Chandrasekar et al., 2014 in India; Basal et al., 2013 in India; Chandrasekar et al., 2011 in India; Havaric et al., 2010 in Bosnia). A lower value of 0.7 was also reported by da Silva (2015). The wide variation observed in the results reported by several investigators may be largely due to the variation in the concentrations of genotoxic chemicals in the cigarette smoke, intensity of cigarette smoking and age group (duration of smoking) of smoker, and other non-smoking factors such as use of alcohol, drugs and life style as also observed by Wu et al. (2004). Although the increase in micronuclei frequency in peripheral blood Lymphocyte and buccal epithelial cells due to cigarette consumption was reported by several researchers from different countries, the present study is first of this kind in generating the data base for Bankura District on the evaluation of genotoxic impact of cigarette smoking using micronuclei frequency in peripheral blood cells and buccal epithelial cells as biomarkers.

Suggestion

MN frequency in lymphocytes is known to be affected by age, gender and multiple dietary and lifestyle factors (Fench and Bonassi 2011). Many studies have shown that chromosomal damage increases progressively with age (Fenech et al. 1994; Ramsey et al. 1995; Bolognesi et al. 1997; Bolognesi et al. 1997; Wojda and Witt 2003). The reasons for the increase are unclear. In addition to age, several other factors may affect the baseline level of chromosome alterations. By understanding the contribution of biological variation, diet, lifestyle, and genetic background to spontaneous chromosomal damage rate, it would presumably be easier to detect the effect of environmental exposures (Fenech 1998). More work is necessary to understand properly how age, gender, lifestyle and other factors affect on MN frequency.

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