Assessment of Smoke Induced Genotoxicity in Sprague Dawley Rats

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Abstract: This study aimed to investigate the genotoxic effect of cigarette smoking in Sprague Dawley rats. About 70 healthy Sprague Dawley rats were randomly divided into two groups at National Institute of Health (NIH). Group-I rats were not exposed to cigarette smoke while Group-II rats were exposed to cigarette smoke for 3 months. Cytokinesis block micronucleus assay (CBMN) was done to determine genotoxicity. Data were subjected to statistical analysis using independent sample t-test. The results revealed that the mean frequency of micronuclei in peripheral blood lymphocytes of control and smoker groups was 3.26 ± 0.741 and 3.63 ± 0.877 respectively. The difference was not statistically significant (p = 0.06). In conclusion, the mean micronuclei frequency in peripheral blood lymphocytes of the smoke exposed rats is higher than that of the control but the difference is not significant.

Key words: Cigarette Smoking • Micronuclei • Genotoxicity

INTRODUCTION

Tobacco smoke remains the main preventable cause of mortality and morbidity worldwide [1-3]. Low and middle income countries are the most severely affected [4]. According to a report, the prevalence of smoking in Pakistan is 23% [5]. Tobacco smoking contributes a significant share to human disease and is a huge economic burden worldwide [2]. Globally more than 5 million people per year die from tobacco use [1, 5, 6]. Smoking is responsible for multitude of ailments in humans [7] and is strongly associated with a variety of human cancers development [8]. Smoking greatly increases the risks of several cancers including those of the lung, esophagus, larynx, mouth, throat, kidney, bladder, pancreas, stomach and cervix [9]. Therefore it is important to understand the mechanism by which smoking increases vulnerability to these diseases as it may open up new avenues for prevention or treatment of complex smoke related disorders [10]. Cigarette smoke is an aerosol containing more than 60 carcinogens [11, 12] such as polycyclic aromatic hydrocarbon, N-nitrosoamines and heavy metals (lead, cadmium) [6, 13] which play a role in cancer initiation and promotion. Tobacco smoke exposure is the main source of genotoxic carcinogens and an important risk factor for several types of cancers in humans nowadays [14]. Exposure to tobacco smoke or its constituents triggers a cascade of events in the multistage process of carcinogenesis. Chromosome aberrations arise due to defects in DNA repair in response to exposure to the clastogenic agent. Chromosome aberrations can be detected through cytogenetic assays such as chromosomal aberrations (CAs), micronucleus (MN) and the comet assay, which have been used as end points of exposure to genotoxic agents [15, 16]. The cytokinesis-block micronucleus (CBMN) assay in human lymphocytes is sensitive, specific and one of the most frequently used cytogenetic methods for measuring DNA damage (i.e., chromosome aberrations) [12, 17]. The CBMN assay is a multi-endpoint assay. It measures not only chromosome damage (i.e., micronuclei (MN); nucleoplasmic bridges (NPB) and nuclear buds (NBUD)), but also other cellular events (such as apoptosis and necrosis) [15, 17]. Micronuclei frequency determination offers several advantages compared to other cytogenetic assays including speed and ease of analysis, no requirement for metaphase cells [18] and reliable identification of cells that have completed only one nuclear division [15, 17]. Furthermore, it has been found that an increased MN frequency in peripheral blood
lymphocytes predicts the risk of cancer in humans [16]. Micronuclei are small nuclei enclosed within a nuclear membrane [19]. They are very similar to cell's nucleus and are the result of chromosomal alterations. Risk of chromosomal alterations becomes higher with increasing micronuclei number [14, 20]. They originate from chromosome fragments or whole chromosomes which fail to be segregated to the daughter nuclei during mitosis as they fail to engage with the mitotic spindle [12, 15, 19]. They may be induced by different factors as exposure to agents (tobacco smoke), oxidative stress and genetic defects in the cell cycle [19]. CBMN assay in peripheral blood lymphocytes is the most reliable method for scoring MN and is standard and most commonly used method [15] to assess both chromosomal damage and genome instability [19]. The present study aimed to evaluate cigarette smoke induced genotoxicity using CBMN assay.

**MATERIALS AND METHODS**

**Study Population:** It was a randomized control trial conducted at Department of Biochemistry & Molecular Biology, Army Medical College Rawalpindi in collaboration with Armed Forces Institute of Pathology (AFIP) Rawalpindi, National Institute of Health (NIH), Islamabad and Center for Research in Experimental and Applied Medicine (CREAM), Army Medical College, Rawalpindi. Approval was taken from Ethics Research Committee of Army Medical College. About 70 healthy Sprague Dawley rats weighing 220 ± 30 g and between ages of 6-8 weeks were obtained from NIH, Islamabad. Diseased rats at the time of study were not included. Animals were kept at animal house under standard conditions with a daily photoperiod of 12 hours light and 12 hours dark. Rats were randomly distributed among the following groups.

**Group I (Control, number = 35)**

Animals in this group were provided with normal pellet diet (NPD) and water ad libitum for a period of 12 weeks. This group was not exposed to smoke.

**Group II (Smokers, number = 35)**

In addition to diet and water this group was exposed to smoke (10 cigarettes/day for 12 weeks). Blood sample measuring 8 – 9 ml blood was collected by intra cardiac puncture from each rat at the end of study. About 3 ml blood was poured into the lithium heparinized tubes and stored at 4°C for estimation of micronucleus frequency by Cytokinesis block micronucleus (CBMN) assay.

**Cytokinesis Block Micronucleus Assay:** Genotoxicity was assessed by cytokinesis-block method described by Fenech [21]. Whole heparinized blood samples were obtained from the rats of both groups. 0.5 ml of whole blood was added into 5 ml of complete medium RPMI 1640 for cultivation of cells. All cultures were incubated at 37°C up to 72 hours. Cytochalasin B at final concentration of 6 mg/ml was added to 44 hour of the culture of lymphocytes, according to the method of Fenech and Morley [21]. The cultures were incubated for another 28 hours. At the end of the incubation period, cultures were harvested. They were treated with prewarmed hypotonic solution (0.075 M KCl) for a few minutes at room temperature. The cell suspension was then fixed in methanol: glacial acetic acid (3: 1) three times. Fixed cells were dropped onto cold microscope slides and air-dried. The slides were stained with 5% Giemsa solution for 5 to 7 minutes. Slides were coded and scored blind under a magnification of 400 X according to the criteria described by Fenech [22]. The MN frequency was determined by analyzing 1000 binucleated cells (BN) per subject [23].

**Data Analysis:** All data were analyzed by SPSS version 17 and represented as mean ± standard deviation. The statistical differences between the smoker and nonsmoker groups were calculated by using student t test. Significant p value was < 0.05.

**RESULTS**

The comparison of mean micronuclei frequency between two groups at the end of 12 weeks is shown in table 1. The mean frequency of micronuclei in peripheral blood lymphocytes was 3.26 ± 0.741 for control group rats while the mean frequency of micronuclei in peripheral blood lymphocytes for group II rats was 3.63 ± 0.877. The difference between control group and group II was not statistically significant (p = 0.06). The mean micronuclei frequency was not significantly increased in exposed as compared to non-exposed rats.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group I (n = 35)</th>
<th>Group II (n = 35)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MN frequency</td>
<td>3.26 ± 0.741</td>
<td>3.63 ± 0.877</td>
<td>0.06</td>
</tr>
</tbody>
</table>
DISCUSSION

In this study the effect of low grade smoking on micronuclei frequency has been evaluated. The micronuclei are the result of genomic damages to the cells. The micronuclei assay is a non-invasive and simple technique for evaluating the DNA damages [24]. The micronuclei assay (MN) has gained increasing popularity as an in vitro genotoxicity test as in comparison with chromosomal aberrations, the scoring of MN is simpler, requires shorter training and is less time consuming [25]. Different forms of smoking such as cigarette, Betel nut and Quid are associated with increase in the number of micronucleus in exfoliated buccal mucosa cells [14, 26-28]. Positive correlation was observed between smoking and micronucleus frequency in the study by Nefic et al [29]. The statistically significant increase in micronuclei frequency was observed in smokeless tobacco chewers as compared to smokers and controls and in smokers than controls by Bansal et al [30]. Mr P et al [31] on the other hand showed that MN count using Micronucleus assay in exfoliated buccal mucosal cells in smokers group was 2.6 times more compared to normal controls [31]. Nefic H et al [29] showed that micronucleus frequency is positively correlated with smoking: the frequency is higher in male subjects with a smoking habit than in female smokers [29]. Zamani AG et al [32] showed that the mean MN frequencies in buccal mucosa, urothelial exfoliative cells and peripheral blood lymphocytes were significantly higher in smokers than in those of controls (P<0.05) [32]. The obese rats exposed to tobacco cigarette smoke presented higher DNA damage compared to control and obese rats exposed to filtered air in a study carried out in 2013 [33].

Despite a few reports showing a positive association, the majority of studies did not find any association between levels of MN frequency and smoking [34, 35]. According to our study, the mean number of micronuclei in peripheral blood lymphocytes of smokers was higher than that of control group rats but the difference was not statistically significant. The result was compatible with the previous studies as no significant difference in the frequency of micronuclei was shown among smokers and non-smokers in a study by Oliveira et al [36]. Another study also showed no statistically significant difference in the frequency of micronuclei between smokers and non-smokers [37]. No statistically significant effect was found of smoking on MN frequency by Coskun et al [38]. No significant correlations were found between duration and intensity of smoking and frequency of micronuclei in lymphocytes by Haveric et al [28].

In conclusion, the mean micronuclei frequency in peripheral blood lymphocytes of the smoke exposed rats is higher than that of the control but the difference is not significant.

REFERENCES


