

## ASSESSMENT OF SMOKE INDUCED GENOTOXICITY IN SPRAGUE DAWLEY RATS

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### ABSTRACT

**Objective:** To investigate the genotoxic effect of cigarette smoking in Sprague Dawley rats.

**Study Design:** Randomized experimental study.

**Place and Duration of Study:** Study was conducted at Army Medical College Rawalpindi, from Jan 2013 to Jul 2013.

**Material and Methods:** Seventy healthy Sprague Dawley rats were randomly divided into two groups at National Institute of Health (NIH). Group-I rats were not exposed 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.

**Results:** The mean frequency of micronuclei in peripheral blood lymphocytes of control and smoker groups was  $3.06 \pm 0.639$  and  $6.77 \pm 0.731$  respectively. The difference was not statistically significant ( $p > 0.05$ ).

**Conclusion:** The mean micronuclei frequency in peripheral blood lymphocytes of the smoke exposed rats was higher than that of the control rats however the difference was not significant.

**Keywords:** Cigarette smoking, Genotoxicity, Micronuclei.

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## INTRODUCTION

Tobacco smoke is the preventable cause of mortality and morbidity worldwide<sup>1</sup>. Developing countries are the most severely affected<sup>2</sup>. According to a report, the prevalence of smoking in Pakistan is 23%<sup>3</sup>. Tobacco smoking is a huge economic burden. More than 5 million people die from tobacco use annually worldwide<sup>4</sup>.

Cigarette smoke contains more than 60 known carcinogens such as polycyclic aromatic hydrocarbon, N-nitrosamines and heavy metals which play a role in several types of cancers in humans. Cigarette smoke also causes accumulation of tar in tissues<sup>5</sup>. Polycyclic Aromatic Hydrocarbons (PAHs) are particularly important as they are composed of highly lipid soluble and hydrophobic substances with remarkable mutagenic and carcinogenic properties. PAHs in their initial form may not exhibit any carcinogenic properties, but their metabolites are tumorigenic as they have ability to produce DNA adducts and

oxidative DNA damage<sup>6</sup>. Oxidative DNA damage induced by exposure to cigarette smoke may be due to the increased production of reactive oxygen species in the body. With disturbed cellular antioxidant defense system, increased amount of ROS can cause oxidative damage to biomolecules such as DNA, proteins and lipids. DNA damage is the main generator of the process of carcinogenesis<sup>7</sup>.

Smoking is the well-established risk factor of cancers in humans<sup>8</sup>. Risk and mortality associated with smoking is correlated with intensity and duration of smoking. Smoking increases the risk of carcinogenesis of the upper respiratory and digestive tracts i.e. oral, oropharyngeal, laryngeal, esophageal, urinary bladder, pancreas, liver and stomach<sup>9</sup>. 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.

The cytokinesis block micronucleus (CBMN) assay in human lymphocytes is sensitive, reliable and most frequently used cytogenetic method for evaluating DNA damage<sup>10</sup>. CBMN assay has

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several advantages compared to other cytogenetic assays as it is rapid, easy to perform and it does not require metaphase cells<sup>11</sup>. MN frequency in peripheral blood lymphocytes has also been used to predict the risk of cancer in humans<sup>11</sup>.

Micronuclei are extra nuclear cytoplasmic bodies enclosed within a membrane<sup>12</sup>. They arise as the result of chromosomal damage. High MN frequency reflects increased chromosomal damage. Therefore, micronuclei are highly recognized as an endpoint for the evaluation of DNA damage<sup>12</sup>. Different factors may induce the formation of MN as tobacco smoking, oxidative stress and genetic defects in the cell cycle<sup>13</sup>.

Several studies have evaluated the genotoxic effect of smoking using different genotoxic assays. However there are only few studies that have assessed the DNA damage induced by passive smoking. The present study therefore aimed to evaluate cigarette smoke induced genotoxicity using CBMN assay in rats thus to assess the genotoxic effect of passive smoking.

## **MATERIAL AND METHODS**

The study was a randomized controlled 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 Ethical Committee of Army Medical College. Seventy healthy Sprague Dawley rats weighing  $220 \pm 30$  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 in the animal house of NIH and were given regular diet and water ad libitum throughout the experiment. The room was properly ventilated with daily photoperiod of 12 hours light and 12 hours dark. It was ensured to maintain room temperature at  $23 \pm 2^\circ\text{C}$  by central temperature regulating system.

Non probability convenience sampling technique was used in the study.

Rats were randomly distributed among the following groups by random number table.

The animals were divided in two groups.

Group I: (control, n=35) Animals in this group were provided with normal pellet diet and water ad libitum for a period of 12 weeks. This group was exposed to room air instead of cigarette smoke. Group II (smoker, n=35) In addition to diet and water this group was exposed to smoke (10cig/day for 12weeks).

The two groups of rats were kept in separate cages; each cage was labeled with the name of the group. The numbering of rats with the permanent marker on their dorsal body wall was done.

For terminal intra cardiac sampling, the rat cages were brought to the laboratory of animal house of NIH, Islamabad that was well equipped with all necessary facilities to anaesthetize rats. Each rat was taken out of the cage and placed in a closed glass container one by one, having cotton soaked with ether<sup>14</sup>. When the rat got completely unconscious, it was taken out of the container and placed at its dorsal body wall on the dissection board. Lower rib cage and sternal margin were identified and then syringe needle was pierced into the heart. Blood was drawn using 10 ml syringe after intracardiac puncture. Care was taken not to pierce the posterior wall of the heart<sup>15</sup>.

Blood sample measuring 8-9 ml blood was collected by intra cardiac puncture from each rat at the end of study. Three ml blood was poured into the lithium heparinized tubes and stored at  $4^\circ\text{C}$  for estimation of micronucleus frequency by CBMN assay.

## **Cytokinesis Block Micronucleus Assay**

Genotoxicity was assessed by cytokinesis-block method described by Fenech (1993)<sup>16</sup>. Whole heparinized blood samples were obtained from the rats of both groups. Half 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 6µg/ml was added to 44th hour of the culture of lymphocytes, according to the method of Fenech and Morley. 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 (2000). The MN frequency was determined by analyzing 1000

The mean count of micronuclei in peripheral blood lymphocytes was  $3.06 \pm 0.639$  for group I rats while the mean count of micronuclei in peripheral blood lymphocytes for group II rats was  $6.77 \pm 0.731$ . The difference between group I and group II was not statistically significant ( $p > 0.05$ ). The mean micronuclei count was not significantly increased in exposed as compared to non-exposed rats.

## DISCUSSION

In this study the effect of low grade smoking on micronuclei frequency has been evaluated. Results showed that the MN count in peripheral blood lymphocytes of smokers was high as compared to that of control group rats but the difference was not statistically significant ( $p > 0.05$ ). Estimation of micronuclei frequency is an

**Table: Mean MN Count of group I and II.**

Mean $\pm$ SD values of MN Count			
Variable	Group I (n=35)	Group II (n=35)	p-value
Micronuclei Count	$3.06 \pm 0.639$	$6.77 \pm 0.731$	$p > 0.05$

binucleated cells per subject<sup>17</sup>.

MN were classified according to Belien and co-workers<sup>18</sup>. MN met the following criteria: a) consist of nuclear material; b) they are completely separated from the parent nucleus; c) they are small in size having less than 1/3 of the diameter of the parent nucleus; d) they are smooth, oval-or round shaped; e) they are on the same plane of focus; f) they are of the same color, texture and refraction as the main nucleus. Cells with two nuclei were considered to be binucleated<sup>18</sup>.

## Data Analysis

Data were analyzed by SPSS version 17 and represented as mean  $\pm$  standard deviation. The statistical differences between the smoker and control groups were calculated by using student t test. A  $p$ -value  $\leq 0.05$  was considered significant.

## RESULTS

Total 70 healthy Sprague Dawley rats were randomly divided into two groups. The comparison of mean micronuclei count between the two groups at the end of 12 weeks is shown in table-I.

easily available tool for early detection of cytological changes in smokers thus avoiding cancer. The CBMN assay is a non-invasive and simple technique for measuring DNA damage<sup>19</sup>.

Bansal H *et al* observed that MN frequency was statistically increased in smokeless tobacco chewers than in smokers and controls<sup>20</sup>.

Ganapathy *et al.* carried out their study to evaluate genotoxicity in human cells exposed to mainstream and side stream cigarette smoke. According to the results significant DNA damage was noted even after 1 hour of exposure to very low doses of mainstream and sidestream cigarette smoke ( $p < 0.01$ )<sup>21</sup>.

Baldawa *et al* carried out a study in which the study subjects were divided into three groups. Group I was control and groups 2 & 3 were smokers with history of smoking 4-10 & 11-20 cigarettes per day respectively. Results of the study showed that mean MN frequencies in group I, II & III were  $0.13 \pm 0.97\%$ ,  $0.405 \pm 1.04\%$  and  $0.668 \pm 1.27\%$  respectively indicating signi-

ficant association between MN frequency and intensity of smoking ( $p < 0.05$ )<sup>22</sup>.

Significant increase in MN frequency ( $p < 0.05$ ) was observed in buccal mucosal and blood lymphocytes of smokers than in controls by Zamani *et al*<sup>23</sup>.

Despite a few studies showing a positive association, the majority of studies did not find any association between MN frequency and smoking. According to our study, the frequency of MN in peripheral blood lymphocytes of smokers was more than that of control group rats but the difference was not statistically significant ( $p > 0.05$ ). The result was compatible with the previous studies as Oliveira *et al* conducted their study in which study population was divided into three groups; 15 smokers, 16 alcoholic smokers and 20 non-smokers. Smears from oral mucosa were examined for micronuclei frequency determination. Results revealed no significant difference in the frequency of micronuclei ( $p = 0.602$ ) among the three groups. No significant association was found between micronuclei frequency and number of cigarettes consumed per day<sup>24</sup>.

Donmez-Altuntas *et al*. had done their study on ninety six healthy subjects. Results showed no statistically significant difference in the frequency of MN between smokers and non-smokers<sup>25</sup>.

de Assis *et al* carried out a study to assess DNA damage using the comet assay in lymphocytes of smoking and non-smoking mothers and their newborns. A total of 120 subjects were recruited in the study; 40 were active smokers and 40 passive smokers and 40 were non-smokers. Results illustrated no statistically significant difference of DNA damage between smokers and non-smokers. Similar results were detected in newborns. No significant difference was observed between newborns born to smoking mothers and those born to non-smoking mothers. Also, no statistically significant difference in DNA damage was observed between mothers and their respective newborns. On the other hand significant DNA damage was detected in lymphocytes of

active smokers and their newborns when compared to that of passive and non-smoking women. Study thus concluded that smoking during pregnancy causes DNA damage to both mother and child<sup>25</sup>.

Haveric *et al* conducted a study on eighty young subjects. Forty three were smokers and 44 were non-smokers, from Bosnia and Herzegovina. Significantly higher frequency of micronuclei in peripheral blood lymphocytes was observed in smokers ( $p < 0.05$ ). No significant correlations were found for duration and intensity of smoking and frequency of micronuclei in peripheral blood lymphocytes and exfoliated buccal mucosal cells<sup>25</sup>.

## CONCLUSION

The mean micronuclei frequency in peripheral blood lymphocytes of the smoke exposed rats was higher than that of the control rats however the difference was not significant.

## CONFLICT OF INTEREST

This study has no conflict of interest to be declared by any author.

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