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Increased frequency of nuclear anomalies in exfoliated buccal mucosa of cigarette smokers

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Abstract

Smoking causes genetic damage associated with increased risk of oral cancer. Cigarette smoking induces genetic aberrations and micronucleus assay is a great biomarker to study the effect of smoking in human population. The present study was undertaken to detect incidence of nuclear anomalies in the exfoliated buccal epithelial cells of smokers using micronucleus assay. 100 healthy individuals (48 smokers and 52 non-smokers) were chosen randomly for the present study. A significantly high frequency of micronuclei was observed in the smokers ($p < 0.01$) as compared to non-smokers. There was significant difference ($p < 0.05$) in the frequency of binucleate cells and karyolysis depicting increased damage in the smokers. A dose-response relationship was observed between smoking and DNA damage.

Keywords: Buccal mucosa, Cigarette smoking, DNA damage, Micronucleus assay

1. Introduction

Cigarette smoking causes severe health problems, like various respiratory disorders and several types of cancer, mainly oral and lung cancer [1]. Various studies have reported that there is a dose-response relation between smoking and development of oral cancer [2-4]. Cigarette contains several carcinogens which get activated in different tissues and cause the formation of DNA adducts [5]. The micronucleus assay with exfoliated buccal epithelial cells, firstly introduced by Stich *et al.* [6] is a less invasive, and cost effective technique and an important biomarker tool to assess DNA damage in which formation of anomalous nuclei or cell is the end point to spot damage and it has been believed that the number of micronucleus is related to increasing effects of carcinogens [7-8]. The formation of micronuclei and other cellular anomalies from either acentric chromosome fragment or a whole lagging chromosome occurs as a result of chromosome breakage due to unrepaired or misrepaired DNA strand breaks or malsegregation of the chromosomes due to mitotic malfunction [7-10]. Micronucleus assay in the exfoliated buccal epithelia can be used to demonstrate the effect of smoking in human population [8-9, 11-12] and is a great biomarker for early detection of malignancy [13]. The cells are observed for micronucleus and other cytogenetic anomalies like binucleate cell (BN), broken egg (BE), karyolysis (KL) and karyorrhexis (KH). The objective of the present study was to assess genetic damage if any, in the exfoliated buccal epithelial cells of cigarette smokers and non-smokers.

2. Materials and methods

2.1 Subjects

For the present study, a total of 100 healthy male subjects, 48 smokers (mean age 37.5 ± 2.22 years) and 52 non-smokers (mean age 40.15 ± 2.12 years) matched in respect to age, lifestyle and socioeconomic status were taken randomly. The study was conducted in Human Genetics Laboratory, Department of Zoology, Kurukshetra University, during the period March-October, 2014. A detailed questionnaire was filled up from each subject to collect details about the subjects regarding their sex, age, dietary habits, smoking and drinking habits and other parameters regarding their lifestyle. The subjects who were taking alcohol or had any kind of medical history were omitted. An informed consent was obtained from each subject prior sampling. Ethical clearance was obtained from Institutional Ethics Committee, Kurukshetra University, Kurukshetra, for present study.

2.2 Sample collection, staining and scoring

The standard technique of Tolbert *et al.* [14] was followed for micronucleus assay with slight variations. The exfoliated buccal epithelial cells were scrapped gently from the inner cheek of the subjects with a moistened wooden spatula. Then the cells were smeared on to the pre-

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cleaned microscopic glass slides. Two slides were prepared for each individual. The cells were fixed with rectified spirit and brought to the laboratory in ice box. The samples were then hydrolyzed in 1 N HCl at 60 °C for 8 minutes and then stained with 2% aceto-orcein stain. Counterstaining was done with 0.1% fast green solution.

Slides were observed under Olympus CX-41 trinocular microscope at 1000x magnification. At least 1000 cells were examined for each subject, and the cells with cytogenetic anomalies were scored. The criterion of Tolbert *et al.* [14] was followed for scanning cells for micronuclei and other nuclear anomalies. The suspected nucleus is required to meet the following criteria in order to be considered as micronucleus : (a) rounded, smooth perimeter suggestive of membrane; (b) less than third the diameter of the main nucleus, but large enough to discriminate shape and color; (c) staining intensity similar to that of nucleus; (d) same focal plane as nucleus. In addition to MN other nuclear anomalies were also studied like BN; the presence of two similar nuclei with in a cell, BE;

nuclei that appear to be broken but still connected to main nuclei with a thin nucleoplasmic bridge, KH; nuclear disintegration involving loss of integrity of the nucleus and KL; complete nuclear dissolution, in which stainless, ghost-like image of the nucleus remains.

2.3. Statistical analysis

Statistical analysis was done using statistical software SPSS version 16 and the results were analyzed by Student’s t-test and ANOVA with Duncan’s multiple range post-hoc test.

3. Results

The mean age of smokers (n=48) was 37.5±1.55 years while that of non-smokers (n=52) was 40.15±1.48 years. No significant difference was observed regarding the age between the smokers and non-smokers. Frequency of micronuclei was found to be significantly higher in smokers (3.25±0.17) as compared to non-smokers (1.77±0.18).

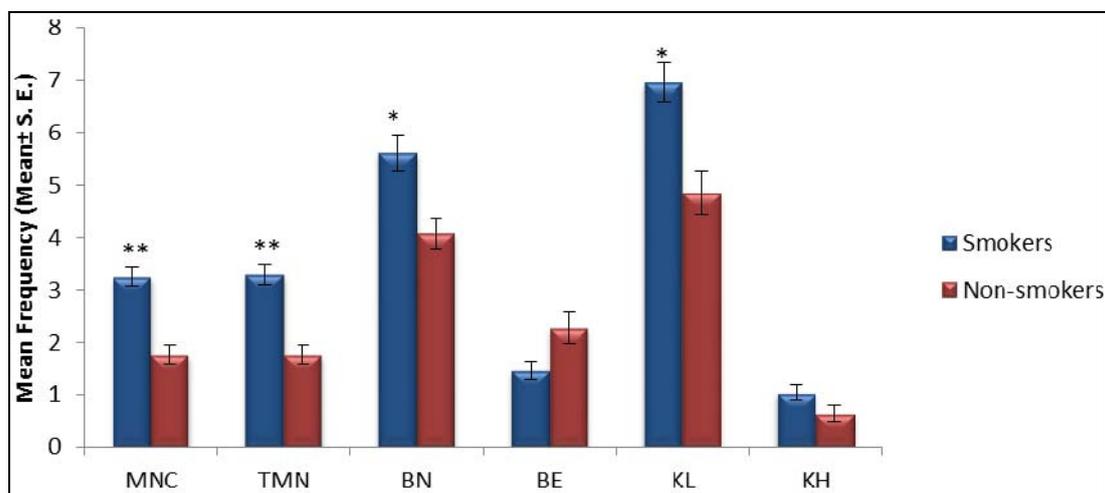


Fig 1: Comparison of nuclear anomalies between smokers and non-smokers
**values are significant (p<0.01), * values are significant (p<0.05), Unpaired Student’s t-test

Similarly, frequencies of BN and KL were also higher in the smokers (p<0.05) in comparison to non-smokers (Figure 1).

The smokers were categorized in 3 groups according to their consumption of cigarettes with group 1 consuming 1-10 cigarettes/day, group 2 consuming 10-20 cigarettes/day and group 3 consuming >20 cigarettes a day. The frequency of

MNC (micronucleated cells) and TMN (Total micronuclei) were observed to be significantly increasing with increasing dose of cigarettes (p<0.05) i.e. in all the groups. The frequencies of BN and KL were found to be significantly higher in group 3 whereas, group 1 and group 2 showed no significant difference (Table 1).

Table 1: Comparison of nuclear anomalies among smokers in relation with various consumptions

Variables	Group 1 1-10 cigarettes/Day	Group 2 10-20 cigarettes/Day	Group 3 >20 cigarettes/Day
N	14	18	16
MNC	2.00±0.00 ^A	3.11±0.08 ^B	4.50±0.29 ^C
TMN	2.00±0.00 ^A	3.11±0.08 ^B	4.62±0.41 ^C
BN	4.57±0.49 ^A	5.11±0.45 ^A	7.12±0.62 ^B
BE	1.57±0.20 ^A	1.33±0.44 ^A	1.50±0.22 ^A
KL	6.43±0.49 ^A	5.56±0.50 ^A	9.00±0.66 ^B
KH	1.14±0.23 ^A	0.56±0.17 ^A	1.50±0.26 ^A

Values with different alphabets in superscript along a row are significantly different (p<0.05), Duncan’s multiple range test

Figure 2 shows the trend of frequency of nuclear anomalies with increasing dose of cigarettes per day. It is clear from the various trend lines that the nuclear anomalies show a dose-

response relation with consumption of cigarettes depicting increased damage with increased consumption.

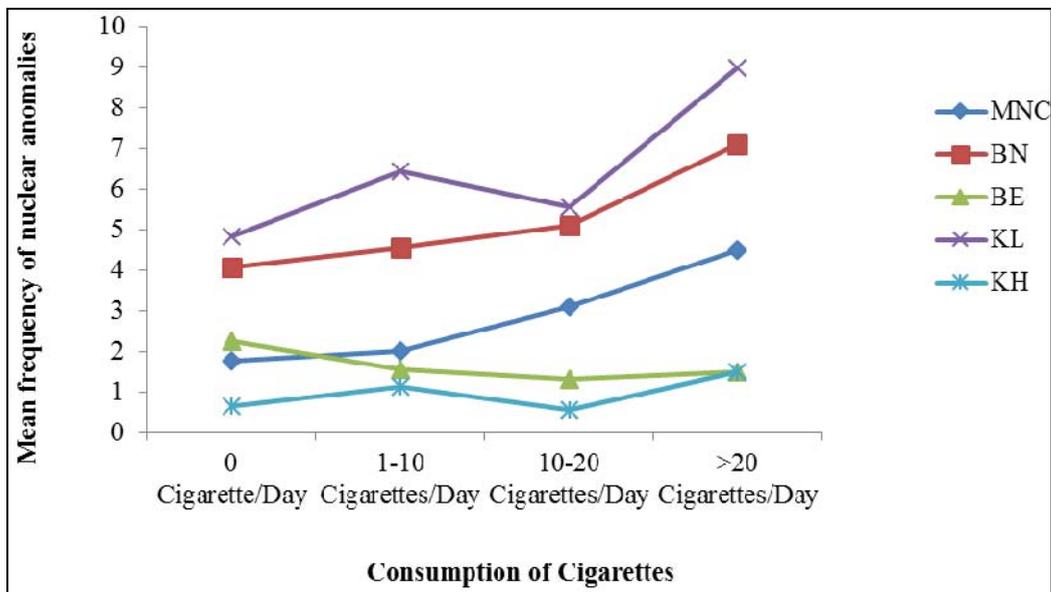


Fig 2: Trend lines show the dose-response relationship of various nuclear anomalies with consumption of cigarettes/day

4. Discussion

The expression of micronuclei in exfoliated buccal mucosa cells are good sources for biomonitoring of genetic damage. The micronuclei assay is a simple, non-invasive technique for evaluating the DNA damage [15]. Many studies have reported that various forms of tobacco and related agents such as cigarette, Betel nut and Quid and reverse smoking are associated with increase in the number of micronuclei in buccal epithelial cells [6, 11, 12, 13, 16, 17, 18].

In the present study the mean frequency of micronuclei in buccal mucosa cells of smokers was markedly higher than nonsmokers ($p < 0.01$). The results are in accordance with the previous studies that have reported the increase in number of micronucleus due to smoking [19-22].

In earlier investigations, cigarette and other forms of tobacco were compared with each other; the subjects were consumers of several different agents. In this study, only the effect of smoking cigarettes on nuclear anomalies has been investigated. None of the earlier studies have considered other nuclear anomalies besides MN. In the present study, we have evaluated the frequencies of other nuclear anomalies also like BN, BE, KL and KH. The frequency of BN and KL were significantly higher in the smokers than non-smokers.

In the present study, none of the subjects were alcohol consumers so, the role of confounding factors like alcohol consumption were omitted. To omit the effect of age and sex on the obtained results, all the samples were men and age-matched. The significant increase in frequency of nuclear anomalies in smokers has been attributed to smoking cigarette. Wu *et al* [21] reported the positive relation between micronuclei frequency and smoking intensity. In this study, the micronuclei frequency in buccal cells was also found to be markedly higher in heavy smokers (consuming >20 cigarettes a day) than light to moderate smokers (consuming 1-10 cigarettes a day). Similar results were obtained for BN and KL frequency, which means there is a positive relation between smoking and genetic damage. Also a dose response relationship was obtained between the consumption of cigarettes and frequency of nuclear anomalies.

Several reports have shown that cigarette smoking and other forms of tobacco consumption increase the frequency of micronucleus in exfoliated buccal epithelial cells [18, 19, 20, 21, 22, 23, 24] but contradictions are also there. Stich and Rosin [8] observed increase in the MN frequency in the alcohol drinkers

who were consuming 2-4 packs of cigarettes a day. Those who were consuming cigarettes alone did not show any significant rise in the MN frequency. This inconsistency can be the result of using different staining procedures, distinction in size of studied population and use of different smoking and smokeless agents.

5. Conclusion

The results of present study strengthen the understanding of possible relationship between smoking and chromosomal damage and also the fact that MN assay in exfoliated buccal mucosa is a useful tool for genotoxicity testing.

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