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Evaluation of MN and other nuclear anomalies in smoker COPD subjects

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Abstract
The present study was carried out to analyse the frequency of micronuclei (MN) and other nuclear anomalies in smoker COPD subjects. For the present study, a total of 110 COPD subjects were analysed out of which 77 were males (mean age 57.299±1.584 years), 33 females (mean age 55.424±1.630years), 49 smokers (mean age 57.347±1.954 years), 61 non-smokers (mean age 56.246±1.528 years) matched with respect to age, lifestyle and socioeconomic status with 90 controls out of which 63 males (mean age 52.444±1.974 years), 27 females (mean age 56.518±2.753 years), 31 smokers (mean age 50.968±2.717 years) and 59 non smokers (mean age 55.084±1.997 years). Nuclear anomalies were analysed using micronucleus assay. In smoker COPD subjects micronuclei (MN) frequency was significantly $p<0.001$ increased along with that the frequency of binucleate (BN), broken egg (BE), karyolysis (KL) and karyorrhexis (KH) were also increased. In case of KH the significant difference was observed at $p<0.01$.

Keywords: COPD, smokers, micronuclei, binucleate, karyolysis, karyorrhexis.

Introduction
Chronic Obstructive Pulmonary Disease (COPD) is a disease characterized by chronic airway inflammation, lung parenchymal inflammation and destruction resulting obstruction in the expiratory airflow. COPD remains a major health problem. It is estimated that in 2020, COPD is projected to rank fifth worldwide in burden of disease according to a study published by the World Health Organization [1]. COPD kills half a million people every year in India, more than those who die due to tuberculosis, malaria or HIV-AIDS [2]. The most prominent risk factor for COPD is tobacco smoking. Skeletal muscles in individuals with COPD generate free radicals at rest, and production increases during muscle contractile activity [3]. Overproduction of free radicals may result in oxidant–antioxidant imbalance in favor of oxidants [4]. The formation of reactive oxygen species (ROS) by the cigarette smoke and inflammatory cells which were generated in the pulmonary epithelial tissues of lungs has been associated with slow growing and irreversible decrease in forced expiratory volume in one second (FEV1), loss of muscle mass and muscle dysfunction [5, 6]. Apart from cigarette in India people smoke tobacco using bidis, hookahs and chillums. Bidis contains one fourth the amount of nicotine as cigarettes but they produce four to five times more tar than cigarettes. So bidis are more harmful than cigarettes and chillum is the most harmful among all of these [7]. Different compounds in cigarette smoke can react directly with cellular components to form radicals, and other procarcinogen substances may get activated to produce single and double strand breaks into DNA [8]. Some recent evidences suggest that the DNA damage/repair imbalance may contribute to increased risk of lung carcinoma in COPD subjects [8]. The micronucleus (MN) assay in buccal epithelial cells is a useful method for detection of unstable chromosomal aberrations [9, 10, 11, 12] and it is a minimally invasive method for monitoring genetic damage in humans [13]. It can be used to reveal the effect of smoking in human population [14, 15, 16, 17]. MN is a small extranuclear DNA particle formed when chromosome fragment or acentric chromosomes lag behind and fails to be included in the main nuclei of daughter cells [18].
The present study evaluate the mean frequency of micronuclei and other nuclear anomalies viz. binucleate cell (BN), broken egg (BE), karyolysis (KL) and karyorrhexis (KH) in COPD subjects and controls.

Materials and methods
Subjects
For the present study, a total of 110 COPD subjects were analysed out of which 77 were males (mean age 57.299±1.584 years), 33 females (mean age 55.424±1.630years), 49 smokers

(mean age 57.347±1.954 years), 61 non-smokers (mean age 56.246±1.528 years) matched with respect to age, lifestyle and socioeconomic status with 90 controls out of which 63 males (mean age 52.444±1.974 years), 27 females (mean age 56.518±2.753 years), 31 smokers (mean age 50.968±2.717 years) and 59 non smokers (mean age 55.084±1.997 years). COPD was diagnosed according to the Global Initiative for Chronic Obstructive Lung Disease guidelines (2013), using clinical history, physical examination, and confirmation by airflow obstruction which is defined as a ratio of forced expiratory volume in one second (FEV1) to forced vital capacity (FVC) below 70% of predicted value. The study was carried out in Human Genetics Laboratory, Department of Zoology, Kurukshetra University, during the period January-August, 2015. A detailed questionnaire was used for each subject to collect details about the subjects regarding their sex, age, smoking and drinking habits, dietary habits, and other parameters regarding their lifestyle. An informed consent was taken from each subject prior sampling. Ethical clearance was obtained from Institutional Ethics Committee, Kurukshetra University, Kurukshetra (No.IEC/14/371) for the present study.

Technique

The standard technique of Tolbert *et at.* (1992) [19] was followed for micronucleus assay (MN). The mouth was rinsed thoroughly with water to remove any unwanted debris. Exfoliated buccal epithelial cells were collected gently from the inner cheek of the subjects with the help of a wooden spatula. Then the cells were smeared on to the cleaned microscopic glass slides and cells were fixed with rectified spirit and brought to the laboratory in ice box then the slides were stained with 2% Aceto-orecin (HIMEDIA, acetic acid RM5564, orcein RM277) for 20 minutes at 40ºC and washed in ethanol and distilled water, respectively. The slides were counter-stained with 0.1% fast green solution (HIMEDIA RM 4266) for 12 minutes and rinsed in ethanol and distilled water, respectively for two to three times and air dried. For each subject 1000 cells were scored under Olympus trinocular microscope (CX-41) at 1000 X magnification. The abnormal nucleus is required to meet the following criteria in order to be selected as micronucleus: (a) rounded, smooth perimeter; (b) should have less than third the diameter of the main nucleus, but large enough to discriminate shape and color; (c) staining intensity should be similar to that of nucleus; (d) same focal plane as main nucleus. Other nuclear anomalies were also studied like Binuleate (BN) i.e. presence of two similar nuclei with in a cell, Broken egg (BE) i.e. nuclei that appear to be broken but still connected to the main nuclei with a thin nucleoplasmic bridge; Karyolysis (KL) i.e. dissolution of nucleus; karyorrhexis (KH) i.e. nuclear disintegration involving loss of integrity of the nucleus.

Statistical analysis

Statistical analysis was carried out using statistical software SPSS version 16 and the results were analyzed by Student's t-test and ANOVA followed by Duncan's multiple range post-hoc test.

Results

Nuclear anomalies were evaluated with the help of Micronucleus assay (MN) in COPD subjects and control subjects. The Mean frequency of MN and other nuclear anomalies BN, BE, KL and KH in COPD subjects were found significantly higher $P<0.001$ as compared to the control subjects (Figure 1). Both control and COPD subjects were divided into non-smoker and smokers groups. In non smoker group, significant difference was observed in the mean frequency of BN, BE, KL and KH in COPD subjects when compared with control subjects (Table 1). The significant difference was observed at $P<0.001$ in case of BN, BE, KL and at $P<0.01$ in KH. But In case of smoker group, MN frequency were also significantly $p<0.001$ increased along with the BN, BE, KL, KH in COPD subjects as compared to the control subjects in case of KH the significant difference was observed at $p<0.01$ (Table 2). COPD subjects and controls were also categorized according to the number of cigarettes consumed daily i.e. 0 cigarette/day, 1-6 cigarettes/day and >6 cigarettes/day. The frequency of MN and BN in control subjects consuming >6 cigarettes/day was significantly higher $P<0.05$ as compared to the non-smokers and subjects consuming 1-6 cigarettes/day. While the mean frequency of BE and KL in control subjects who have consumed >6 cigarettes/day were significantly higher $P<0.05$ as compared to the non smokers only (Table 3). Similarly the mean frequency of MN, BN, were significantly higher $p<0.05$ in COPD subjects consuming >6 cigarettes/day as compared to the 0 cigarette/day consumers and those consumed 1-6 cigarettes/day and the frequency of BE and KL were significantly higher ($p<0.05$) in smokers consuming > 6 cigarettes/day as compared to the non smokers (Table 4).

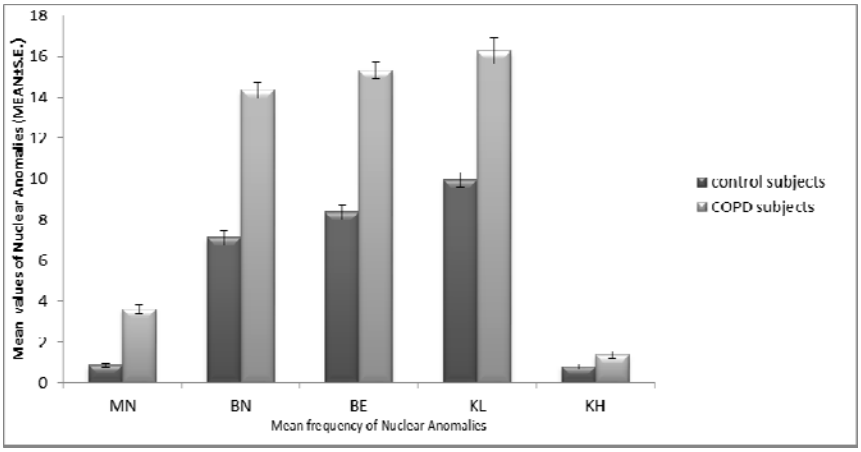

Fig 1: Frequency of nuclear anomalies in control subjects and COPD subjects.

Table 1: Comparison of nuclear anomalies (Mean±S.E.) in control subjects (n=90) and COPD subjects (n=110).

S. No.	Nuclear Anomalies	Subjects	Mean±S.E.
1	MN	Control	0.867±0.115
		COPD	3.618±0.238***
2	BN	Control	7.122±0.355
		COPD	14.354±0.404***
3	BE	Control	8.356±0.339
		COPD	15.327±0.425***
4	KL	Control	9.956±0.350
		COPD	16.264±0.634***
5	KH	Control	0.811±0.120
		COPD	1.372±0.187**

***Significant (P<0.001; 2-tailed) (Independent sample t test)
**Significant (P<0.01; 2-tailed) (Independent sample t test)

Table 2: Comparison of nuclear anomalies (Mean±S.E.) of non-smokers (n=120) and smokers (n=80) of both control and COPD subjects.

Nuclear Anomalies	Non-smokers		Smokers	
	Control (59)	COPD (61)	Control (31)	COPD (49)
MN	0.695±0.133	2.951±0.295	1.193±0.209	4.449±0.358***
BN	6.441±0.448	12.541±0.367***	8.419±0.514	16.612±6.658***
BE	7.864±0.451	13.344±0.474***	9.290±0.440	17.796±0.832***
KL	8.847±0.407	11.541±0.443***	12.064±0.466	22.143±0.666***
KH	0.898±0.169	1.147±0.237**	0.645±0.135	1.653±0.297*

***Significant (P<0.001; 2-tailed) (Independent sample t test)
** Significant (P<0.01; 2-tailed) (Independent sample t test)
*Significant (P<0.05; 2-tailed) (Independent sample t test)

Table 3: Comparison of nuclear anomalies (Mean±S.E.) with respect to number of cigarettes consumed daily in control subjects (n=90).

Nuclear Anomalies	Smoking habit		
	0 cigarette/day (59)	1-6 cigarettes/day (16)	>6 cigarettes/day (15)
MN	0. 695±0.134^{A}	0.562±0.182 ^{A}	1.867±0.306 ^{B}
BN	6.441±0.448 ^{A}	7.562±0.785 ^{A}	9.33±0.591 ^{B}
BE	7.864±0.451 ^{A}	8.750±0.412 ^{B}	9.714±0.1.267 ^{B}
KL	8.847±0.407 ^{B}	12.875±0.724^{AB}	11.200±0.509 ^{A}
KH	0.898±0.169^{A}	0.750±0.214 ^{A}	0.533±0.165 ^{A}

Means with different letter in superscript (A, B, AB) in the same row are significantly (P<0.05) different (Duncan's post hoc test).

Table 4: Comparison of nuclear anomalies (Mean±S.E.) with respect to number of cigarettes smoked daily in COPD subjects (n=110).

Nuclear Anomalies	Smoking habit		
	0 cigarette/day (61)	1-6 cigarettes/day (22)	>6 cigarettes/day (27)
MN	2.950±0.295^{A}	2.954±0.375 ^{A}	5.667±0.459 ^{B}
BN	12.541±0.367^{A}	13.955±1.007 ^{A}	18.778±0.616 ^{B}
BE	13.344±0.474 ^{A}	17.545±0.860 ^{B}	18.000±0.806 ^{B}
KL	11.540±0.445 ^{A}	21.818±1.139 ^{B}	22.407±0.790 ^{B}
KH	1.147±0.673 ^{A}	2.364±1.308 ^{B}	1.074±0.320 ^{A}

Means with different letter in superscript (A, B) in the same row are significantly (P<0.05) different (Duncan's post hoc test).

Discussion

COPD is influenced by genetic factors, common infection of airways and genotype-environmental interactions [20, 21]. The major risk factor for COPD, particularly in developed countries, is cigarette smoking [22, 23]. Cigarette smoking is the major environmental determinant of COPD [24, 25]. Cigarette smoke contains more than 1014 free radicals per puff and it is a complex mixture of over 4700 chemical compounds, including quinones, semiquinones, nitrosamines, aldehydes, benzopyrene, and other carcinogens, and it is a risk factor in the development of COPD and lung cancer [26, 27, 25]. In recent studies strong epidemiological relationship has been found between tobacco smoking and increased risk of cancer, together with experimental studies, suggest that carcinogenic aromatic amines, polycyclic aromatic hydrocarbons, nitrosamines, and additional toxic compounds in cigarette smokers reach target cells to exert their carcinogenic and co-carcinogenic actions [28]. Buccal epithelial cells are the first barrier for the ingestion and inhalation route and are capable of metabolizing imminent carcinogens to reactive elements [29, 30, 31, 32]. Approximately 90% of human cancers arise from epithelial cells [33]. Recent studies have reported that various forms of tobacco consumption are associated with increase in the frequency of micronuclei in buccal epithelial cells [34, 35, 36, 37, 38, 39]. In the present study results of micronucleus assay also indicate an increase in the frequency of MN and other nuclear anomalies like BN, BE, KL and KH in COPD subjects than controls. Maluf *et al*. (2007) [3] also reported the increased frequency of micronuclei and binucleate cells in COPD subjects than controls. While Casella *et al*. (2006) [40] studied the frequency of micronuclei in peripheral blood and reported no genotoxicity in patients with COPD. Our results on COPD smoker subjects and control smoker subjects also demonstrated an increase in frequency of MN and other nuclear anomalies in smokers consuming (>6 cigeratte/day) than non smokers. The increased frequency of micronuclei and other nuclear anomalies in COPD subjects primarily assigned to clastogenic events and DNA amplification. The present study is the first one to evaluate the nuclear anomalies like BE, KL and KH along with MN and BN in COPD subjects. In conclusion, our results showed that smoker COPD subjects have significantly elevated frequency of nuclear anomalies, as detected by the MN assay, as compared to the control smoker subjects.

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