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MICRONUCLEI AND SISTER CHROMATID EXCHANGES IN WOMEN BEEDI WORKERS OCCUPATIONALLY EXPOSED TO TOBACCO DUST

Rudrama Devi.K* and Minny Jael.P

Human Genetics and Molecular Biology Lab, Department of Zoology, Osmania University, Hyderabad,India-500007.

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Corresponding Author:
Rudrama Devi.K
Human Genetics and Molecular Biology Lab, Department of Zoology, Osmania University, Hyderabad,India-500007.

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ABSTRACT

To investigate the genotoxicity of tobacco dust in tobacco industry women, a study was conducted on a group of employees in a tobacco factory, was tested for micronuclei (MN) and sister chromatid exchanges (SCE) and are related to the hematological parameters that are well established as indicators of early biological effects. A total of 60 women bidi workers and 60 control groups of individuals in the age group of 16 to 65 years and 6-30 yrs of exposure were recruited; a questionnaire based survey was conducted. The mean levels of the differential leukocyte count were significantly higher in beedi rollers when compared with the controls and buccal smears were collected from oral cavity and analyzed for nuclear anomalies. A higher frequency of micronuclei was observed among women exposed to tobacco dust than normal controls. A significant increase in nuclear anomalies was observed in workers exposed to tobacco dust for longer duration. In addition to this, a higher degree of sister chromatid exchanges in blood lymphocytes were observed among tobacco exposed women.

INTRODUCTION

India is the third largest producer of leaf tobacco in the world; it is also a very large consumer of tobacco products. More than five million individuals are involved in the production of beedis in India. Typically, an individual beedi may contain roughly 0.2 to 0.5 grams of pulverized sun- cured locally grown tobacco in a tendu or temburi leaf obtained from native plants, Diospyros melanoxylon or Diospyros ebenum respectively. The sun dried tobacco filler is hand rolled using a leaf cut into a cylindrical shape and secured with a thread. These beedi rollers work in small factories or at household- base enterprises in an environment laden with tobacco dust. Individuals working 6 to 10 hr/day inhale, swallow and explore their skin and mucous surface to significant amounts of particulate tobacco.

Beedi rollers are exposed to unburnt tobacco, mainly through the cutaneous and nasopharyngeal routes. The constituents of tobacco get absorbed into the body, get bioactivated and results in increased risk of developing ailments for which tobacco consumption is a major risk factor. Beedis contain many potentially harmful chemical constituents, including carcinogenic chemicals such as the tobacco specific nitrosamines (TSNAS), polyaromatic hydrocarbons (PAHs), aromatic amines, phenols and metals. In addition to harmful chemical constituents that are native to tobacco, the pressure of others may originate as a consequence of the various additives used to make flavored varieties [1].

During the last few years, genotoxicity biomarkers have received considerable interest as tools for detecting human genotoxic exposure and effects, especially in health surveillance programs dealing with chemical carcinogens. There was only less attention to occupational exposure to tobacco and their hematological parameters. Blood is a part of the circulatory system of the body and it has several functions. Much valuable information can be readily obtained from blood parameters. A wide variety of diseases and other dysfunctions may show signs or symptoms of hematological diseases like esonophilia, anemia and are highly associated with different food habits, lifestyle and environmental hazards [2].

Furthermore an increased cytogentic damage in peripheral blood lymphocytes of workers occupationally exposed to tobacco dust has been demonstrated using different genetic end points, such as sister chromatid exchanges (SCE) and micronucleus (MN) [3,4]. Micronucleus test in exfoliated buccal cells has been shown to be an effective method to be an effective method to detect unstable chromosomal aberrations [5]. Human population exposed to the toxic chemicals present in tobacco dust such as benzene showed a significant increase in the buccal cell micronuclei [6]. Buccal epithelial cells provide an alternate source of tissue in the human subjects monitoring for occupational and environmental toxic exposures. On the other hand the induction of SCE has been widely used as an indicator of DNA damage following

exposure to pesticides which is one of the 4000 chemicals present in tobacco.

Hence the objective of the present investigation is to study the extent of hematological changes, cytogenetic damage in exfoliated buccal cells and SCE in women beedi rollers. Degenerative nuclear changes such as Micronuclei (MN), Binucelates (BN), Nuclear bridges (NB) and Karyolytic cells were analyzed in the exfoliated buccal cells.

MATERIALS AND METHODS:

Study Population:

The study was carried out on 60 women beedi rollers. The control group consists of 60 healthy individuals with no exposure to any potential genotoxic substances. Participants were informed about the objective of the study. They were asked to complete a questionnaire to obtain necessary information on their life style and personal factors (age, working period, smoking habits, health etc). Subjects were chosen such that none of them were smokers or alcoholics.

Blood collection:

Exfoliated buccal cells were obtained from women beedi rollers for investigating micronuclei. Heparinized venous blood samples were drawn for performing sister chromatid and analyzing Hb, RBC, platelets and TLC [7].

Hematological studies:

Polymorphonuclear neutrophils are very important in the body's acute inflammatory process, while lymphocytes, macrophages and monocytes are mononuclear phagocytic cell, are indispensable in the body immune system and chronic inflammatory response [8]. Eosinophils responds to chemotactic substances produced by mast cell when introduced by the presence of persistent antigen-antibody complexes such as chronic parasitic, dermatological and allergic conditions while basophils, whose granules contain a number of preformed mediators of the inflammatory response including histamine and chondrotin sulphate, also stimulate leukotriene and other mediators upon stimulations [9].

Sample collection:

Two milliliter of blood was collected from the cephalic vein, a prominent vein in the cubital *fossa* of both test and control subjects in a fasting condition between 8 am and 10 am daily into sample bottles, which contained Dipotassium salt of Ethylene Diamine Tetra Acetic Acid (EDTA) at a concentration of 2 µg/ mL of blood. Gentle mixing was done immediately to ensure complete anticoagulation of the blood. Hematological parameters were studied according to the methods of Dacie and Lewis [10] included Hemoglobin (Hb), total leukocyte count (TLC) and differential leukocyte counts, red blood cells (RBC) and platelets.

Micronuclei:

Micronuclei are cytoplasmic bodies having a portion of acentric chromosome or whole chromosome which are not carried to the opposite poles during the anaphase. Their diameter may range between 1/3 or 1/6, the diameter of the main nucleus. Binuleated cells have two nuclei that are adherent to each other. This is indicative of failed cytokinesis. Presence of Micronuclei is a biomarker of chromosomal damage or loss. Nucleoplasmic bridges (NPB) are formed due to dicentric chromosomes that originate from either misrepair of DNA breaks or telomere end fusions. Nuclear buds (NBUD) is a biomarker of gene amplification. Karyorrhetic cells have a dense network of nucelochromatin elements that lead to fragmentation and disintegration of the nucleus. In karyolytic cells, the nucleus is devoid of DNA and appears to have no nuclei. This indicates a very late stage in the cell death process. It has a cloudy appearance with no distinct features.

Collection, preparation and staining of exfoliated buccal cell:

Buccal cells originate from multilayered epithelium that lines the oral cavity. Prior to buccal cell collection the bidi rollers and control were advised to rinse their mouth thoroughly with water to remove unwanted debris. Samples were collected using a wooden tongue-depressor, a metal spatula or a Cyto brush moistened with water or buffer to swab or gently scrapes the mucosa of the inner lining of one or both cheeks. The cells were smeared on clean glass slides, kept in phosphate buffer saline (PBS) for 10 mins, fixed with acetic acid:methanol (1:3) and air dried. The slides were stained with giemsa and rinsed with double distill water, air dried and viewed under a light microscope [11].

Sister chromatid exchanges:

Sister chromatid exchange (SCE) refers to the interchange of DNA between replication products. This occurs during S phase and is efficiently induced by mutagens that form DNA adducts or that interfere with DNA replication. The formation of SCEs has been correlated with recombination repair and the induction of point mutations, gene amplification and cytotoxicity. The technique for detecting such exchanges takes advantage of the semiconservative nature of DNA synthesis. To allow for a differential staining that enables the researcher to distinguish both chromatids, BrdU (Bromo-deoxy-uridine) is added to the culture medium for the duration of two complete cell cycles. Chromatids in which only one strand of DNA incorporated BrdU show a normal dark Giemsa staining, whereas those with two substituted strands, stainless darkly. If an exchange occurred, this can be seen as the dark part changes to the other arm: "harlequin chromosomes".

Collection and culture of peripheral blood lymphocytes for sister chromatid exchanges:

Blood samples were collected in disposable pre-sterilized heparinized syringes and transferred to the laboratory without delay for lymphocyte culture. Lymphocyte cultures were initiated with 0.5ml of whole blood in RPMI 1640 medium containing 20% AB serum, 0.5% phytohaemagglutinin and 0.25% antibiotic. The cultures were incubated at 37° C for 72 hours. For sister chromatid exchanges, 5- bromodeoxyuridine (10 Hg/ml, Sigma) was added 24 h after setting up the cultures. Cells were harvested after 72 h. Slides were prepared by air drying method and stained with Hoechst 33258 and 4% Giemsa, following the method [12]. For calculating frequency of SCE per cell, 30 metaphases were analysed as per international practice.

Micronucleus and sister chromatid exchange images were taken under the oil immersion objective using Leica light microscope attached with a high performance CCD camera with a magnification of 100X.

RESULTS

Hematological parameters:

The general characteristics of the study population are presented in Table 1.

Table1: Demographic characteristics:

	Controls		Exposed	
Age groups	No. of individuals	Age Mean ±SD	No. of individuals	Age Mean ±SD
16-25	10	22.7±2.41	9	21.1±2.13
26-35	13	29.10±2.05	14	30.7±2.95
36-45	14	39.06±1.43	13	40.9±2.94
46-55	12	47.8±1.34	16	49±2.84
56-56	11	58.5±1.21	8	60±2.5

P>0.05

The group under study was analyzed based on the age and no difference was observed between the study groups. The mean level of Hb content and TLC of experimental and control groups were represented in Table 2. There was a statistically significant ($p<0.05$) decrease in the value of Hb and increase in the mean values TLC and Platelet were obtained in a women beedi rollers occupationally exposed to tobacco dust when compared with the control group. The mean levels of differential leukocyte count were represented in Table 1. The percentage of Lymphocytes (35.25 ± 0.84), Eosinophils (2.60 ± 0.51) and Basophils (0.86 ± 0.02) Neutrophils (53.67 ± 2.08) and Monocytes (6.16 ± 0.15) were significantly increased in women beedi rollers than in controls.

Table 2: Hematological parameters:

Blood characteristics	Controls (Mean ± SD)	Exposed (Mean ± SD)
Hemoglobin Hb (g/100mL)	14.23 ± 0.65	13.16 ± 1.02*
TLC (10^6 /L)	9160.75 ± 1010.20	9471.06 ± 1192.04*
Neutrophil (%)	54.69 ±1.71	53.67 ± 2.08*
Lymphocyte (%)	32.18 ± 0.73	35.25 ± 0.84 *
Eosinophil (%)	3.30 ± 0.43	2.60 ± 0.51 *
Monocyte (%)	7.38 ± 0.05	6.16 ± 0.15*
Basophils	1.03 ± 0.03	0.86 ± 0.02*
Platelet	238.29 ± 22.31	225.29 ± 21.52*
n	60	60

P<0.05* Hematological results of the study population [All values are given as mean ± S.D. Significant difference between study groups; p< 0.05 controls and women beedi rollers].

Table 3: Micronuclei in control group:

Age groups	No. of individuals	MNC	BNC	KRC	KLC
16-25	10	2.10 ±2.41	3.72±0.40	6.10±1.20	26.42±1.01
26-35	13	2.11±0.08	3.45±0.91	8.62±0.40	22.70±0.12
36-45	14	2.42±0.09	3.10±1.20	8.71±1.34	24.61±1.04
46-55	12	2.70±0.10	4.20±0.48	9.12±1.08	28.10±1.08
56-56	11	3.12±0.17	2.92±0.68	6.80±0.41	26.47±1.00

p>0.05

Table 4. Micronuclei in exposed group:

Age groups	No. of individuals	MNC	BNC	KRC	KLC
16-25	9	4.30±0.02*	3.42±0.47	6.80±1.90	30.14±0.93*
26-35	14	4.21±0.06*	3.61±0.72	8.92±0.32*	33.71.±0.4*
36-45	13	4.83±0.09*	3.91±1.40	8.71±1.34	35.93±0.02*
46-55	16	4.78±0.18*	4.01±0.09	9.73±0.06	38.17±0.45*
56-56	8	5.22±0.02*	3.94±0.07*	7.60±0.60*	39.38±1.03*

P<0.05*

Micronuclei:

Age and their socio-economic status were nearly similar in both the groups as shown in table 1. The age group of the selected workers belongs to the range from 16-25, 26-35, 36-45, 46-55 and 56-65. Mean ±SD values were taken for each group i.e., in controls and exposed as shown in table 1. The results obtained from micronuclei assay, are used to reveal DNA damage in occupationally exposed beedi workers. Micronuclei, binucleated, karyorrhexis and karyolytic cells were higher in women beedi rollers when compared with the controls as shown in table 3 & 4. Results show that nuclear anomalies are significantly higher in women beedi rollers when compared with the controls.

When the analysis was done, taking years of exposure into consideration the Mean±SE was significantly higher in women body rollers who were exposed to tobacco dust for more than 20 years as shown in table 5. The mean values of micronuclei with the increase in exposure time in women beedi rollers were 6.57±0.82 as against 4.80±1.03 in non beedi rollers.

Table 5. Show the main characteristics of the case- controls studied.

Years of exposure	Women beedi workers		
	No. of individuals	Yrs of exposure Mean ±SD	MN (Mean ±SD)
Control 6-30	**60**	**-**	**4.80±1.03**
Exposed 6-10	20	7.2±0.23	3.71±0.39*
11-15	18	12.7±0.34	6.24±0.73*
16-20	9	18±0.47	5.66±0.58*
21-25	7	23.1±0.42	6.22±0.62*
26-30	6	27.6±0.62	7.15±0.05*
6-30	**60**	**22.43±1.89**	**6.57±0.82***

P<0.05*

Sister chromatid exchanges:

The results of the present study showed a significant increase in the incidence of sister chromatid exchanges in women beedi rollers when compared with the control groups as shown in table 6 the percentage of mean SCE rate per cell in the exposed group was 1.76±0.06 as against 0.54±0.43 in the control group. The data on the incidence of SCE were also analyzed duration wise. The incidence of SCE increased as the duration of exposure increased from 0.54±0.43 in the control group to 3.29±1.09, 1.881±0.50, 1.63±0.45, 1.56±0.39 and 2.18±0.77 in groups exposed for 6-10,11-15,16-20,21-25 and 26-30 years respectively.

Table 6. Sister chromatid exchanges:

Years of exposure	No. of samples	Total metaphases scored	Total no. of SCE	Mean ±SD
Control 6-30 yrs	**60**	**6000**	**3240**	**0.54±0.43**
Exposed 6-10 yrs	9	1000	3293	3.29±1.09
11-15 yrs	14	1000	1881	1.881±0.50*
16-20 yrs	13	1000	1638	1.63±0.45*
21-25 yrs	16	1000	1564	1.56±0.39*
26-30 yrs	8	1000	2184	2.18±0.77*
6-30 yrs	**60**	**6000**	**10560**	**1.76±0.06***

P<0.05*

The statistical analysis shows that the t' values for the incidence of SCE between control group and exposed were significant. (P<0.05)

DISCUSSION

The high TLC count represents a primary disorder of leukocyte production or may reflect a secondary response to some disease process or toxins [13]. The peripheral blood leukocyte count is a marker of inflammatory activity

and ongoing tissue inflammation from whatever underlying cause, it might be viewed as a bio-marker of inflammatory response. Longitudinal studies have linked elevations of the peripheral blood leukocyte count to increased mortality from decreased pulmonary function, ischemic heart disease and cancer [14]. Removal of major risk factor such as exposure to tobacco derivatives, a mixture of betel quid, Areca nut, tobacco chewing and smoking could increase healthy life expectancy in every region of the world. The MN test i.e., scientifically approved is important in demonstrating the genotoxic effects of harmful substances on health [15]. Micronuclei in exfoliated epithelial cells are useful biomarkers of occupational exposure to genotoxic chemicals. Increase in exposure to toxic chemicals such as formaldehyde and benzene induces a significant increase in the buccal cell micronuclei [16]. A study showed an increase in the incidences of micronuclei in buccal cells of lead battery unit workers [17]. A high frequency of kayolysis was observed among lead exposed workers [18].

Our results make it clear that woman beedi rollers showed an increased frequency of cells with micronuclei due the genotoxicity effect of tobacco derivatives to which they are exposed. Such significant difference was also noticed in SCE among beedi rollers than controls. The suitable method adopted for studying cytogentic effects induced by a suspecting agent in human beings is the microculturing of human peripheral blood lymphocytes. SCE's can be observed in any cell that has completed two replication cycles in the presence of Brdu induction of cytogenetic damage. Mutagenic investigation is one of the necessary evaluations to be done, to ensure environmental quality and occupational health and workers' education about decreasing genetic damage and risk of serious diseases.

There are several reports showing the increased yields of SCE's in occupational asbestos exposed population [19], rubber chemicals [20], coal miners [21] and industrial painters [22].

Furthermore, increased micronuclei frequency in the grossly normal appearing oral mucosa of higher risk individuals is associated with greater risk of oral cancer development. The present study clearly indicated that occupational exposure to tobacco greatly affects the cell level of Hb, TLC, RBC, platelets, micronuclei and sister chromatid exchanges. Therefore the genetic composition of the total blood count, MN and SCE must be studied to determine if they contain specific genes associated with various cancers. The results of such studies could have a significant impact on the future use to detect DNA damage in any kind of genotoxicity and toxicology biomarker.

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