

INFLUENCE OF DIETARY ASCORBIC ACID INTAKE ON THE INCIDENCE OF MICRONUCLEI AND SISTER CHROMATID EXCHANGES IN WOMEN BEEDI WORKERS EXPOSED TO TOBACCO DUST

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ABSTRACT

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 beedies 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. A population monitoring

study was conducted in human lymphocytes by analysis of micronuclei and sister chromatid exchanges to investigate whether occupational exposure to tobacco dust is genotoxic to women bidi rollers. There was significant increase in the frequency of micronuclei and Sister Chromatid Exchanges in human lymphocytes. In addition, a study on the antimutagenic effect of Vitamin C administered orally to tobacco exposed workers was investigated by measuring the frequency of micronuclei and sister chromatid exchanges. It was found that a three month daily intake of Vitamin C. The results clearly showed a significant reduction in chromosomal aberrations and sister chromatid exchanges frequency evaluated after vitamin-C treatment. These results of present study indicate the protective nature of AA supplementation in tobacco exposed workers and beneficial to compensate hazards due to tobacco exposure.

KEYWORDS: Ascorbic acid, Sister chromatid exchanges, human lymphocytes, lead battery industry workers.

INTRODUCTION

Beedi 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 eosinophilia, anemia and are highly associated with different food habits, lifestyle and environmental hazards.^[2]

Furthermore an increased cytogenetic damage in peripheral blood lymphocytes of workers occupationally exposed to tobacco dust has been demonstrated using different genetic endpoints, 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. Degenerative nuclear changes such as Micronuclei (MN), Binucleates (BN), Nuclear bridges (NB) and Karyolytic cells were analyzed in the exfoliated buccal cells. The protective nature of ascorbic acid supplementation in tobacco induced micronuclei in women bidi rollers in

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.

Subject Recruitment and Sample Collection

The study was conducted on 182 females aged 16-66 years from Wrangal, Nizamabad & Adilabad districts of Telangana. The control groups consisted of 182 healthy females aged 16-66 with no history of exposure to clastogeni and/or aneugenic agents and socio-economic level also similar to that of experimental subjects. At the time of sample collection (3ml/individual) all the bidi rollers signed a term of informed consent and replied to Questionnaires elaborated to determine the profile and habits of study population. The protocol has been approved by local ethical committee. The exposed women to tobacco dust, the duration of service was taken more than five years. Peripheral blood samples ($V = 5$ ml) were collected under sterile conditions by venipuncture into heparinized tubes for comet assay.^[3]

2 Estimation of ascorbic acid

Plasma ascorbic acid levels were measured by an ion pairing HPLC method (Ross 1994) in control and as well as in workers occupationally exposed to tobacco dust.

Table 1: Levels of Ascorbic Acid in women bidi rollers

Groups	No. of samples	Levels of Ascorbic Acid mg/dl
Control	50	0.46 ± 0.07
Women Bidi workers	84	$0.36 \pm 0.06^*$
Group A	46	$0.58 \pm 0.06^{**}$
Group B	38	$1.10 \pm 0.04^{**}$

a) * $P < 0.05$ Significant with control group

Table 2: Characteristic profile of control and women bidi rollers.

Groups	Sample	AA	No.of examines	Age in Years Mean \pm SD	Employment (Years)
Control	Feb 2014	-	50	32.80 ± 0.80	22.95 ± 1.90
	April 2014	+	42	34.70 ± 1.20	22.10 ± 0.80
Exposed	Feb -2014	-	84	36.80 ± 1.20	29.80 ± 1.20
	April - 2014	+	74	38.20 ± 0.06	22.10 ± 0.80

$P < 0.05$

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. Binucleated 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 nucleochromatin 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 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

Table 3: Effect of ascorbic acid for 12 weeks on the incidences of binucleated micronuclei in women bidi rollers (Adilabad district)

Group	Sample timing	Ascorbic acid	No. of examinees	No. of binucleated cells with MN	Percentages of binucleated cells with MN
Control	Feb –2014	-	42	8204	0.68±0.52
	April- 2014	+	40	8080	0.76±0.08
Exposed group A	Feb –2014	Pre	38	7560	3.32±1.12a
	April – 2014	Post	36	7120	1.08±0.06b
Exposed group B	Feb- 2014	Pre	34	6800	3.36±0.82a
	April - 2014	Post	32	6380	0.92±0.96b

*P<0.05

100 binucleated cells were observed per sample

- a- Denotes significance of data with control group
- b- Denotes significance of data with exposed group

Table 4: Effect of ascorbic acid supplementation for 12 weeks on the incidences of SCE in women bidi rollers in adilabad district

Group	Sample	Ascorbic acid	No. of examinees	No. of metaphases	Mean \pm SD
Control	Feb- 2014	-	48	1420	3.40 \pm 0.08
	April – 2014	+	46	1360	3.80 \pm 0.06
Exposed Group A	Feb - 2014	Pre	42	1200	8.20 \pm 1.20
	April - 2014	Post	36	1060	5.20 \pm 0.08
Exposed Group B	Feb - 2014	Pre	36	1020	7.92 \pm 1.06
	April - 2014	Post	34	980	4.20 \pm 1.20

*P<0.05

30 metaphases were for each sample

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 \pm 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 \pm 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 \pm 0.82 as against 4.80 \pm 1.03 in non beedi rollers. 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 \pm 0.06 as against 0.54 \pm 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.

Micronucleus assay in human lymphocytes

The frequency of micronuclei in binucleated cells prior to AA supplementation was 0.68 \pm 0.52 and increased to 0.76 \pm 0.08 after 12 weeks of AA supplementation. However the

differences between the percentages of micronuclei between two groups were found to be insignificant ($P>0.05$).

The percentage of micronuclei in binucleated cells is significantly increased from 0.68% to 3.32% and 3.36 % in tobacco dust exposed workers (Group A & B). However when subjects were given vitamin c there is a decrease in the percentage of micronuclei to 1.08 % in group A 0.92 % in group B respectively. The differences in the percentages of micronuclei between tobacco dust and controls were found to be significant where as the frequency of micronuclei between tobacco dust (prior to AA administration) and AA treated group where showed statistically significant ($p<0.05$).

Sister chromatid exchanges in human lymphocytes

The frequencies of SCE prior to AA supplementation was 3.40 ± 0.08 and increased to 3.80 ± 0.06 after 12 weeks of AA supplementation. However the differences between the percentages of micronuclei between two groups were found to be insignificant ($P>0.05$).

The percentage of SCE is significantly increased from 3.40% to 8.20% and 7.92 % in tobacco dust exposed workers (Group A & B). However when subjects were given vitamin c there is a decrease in the percentage of micronuclei to 5.20 % in group A 4.20 % in group B respectively. The differences in the percentages of SCE between tobacco dust and controls were found to be significant where as the frequency of SCE between tobacco dust (prior to AA administration) and AA treated group where showed statistically 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 58. 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 karyolysis 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 cytogenetic 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]

Many naturally occurring substances in plants and other sources have protective effects on environmental mutagens / carcinogens and also on endogenous mutagens (Ferguson, 23) It has been reported that the common use of antimutagens and anticarcinogens in everyday's life will be the most effective against the genetic and other related diseases Vitamin supplements have a marked potentiality against toxic effects of diversified environmental chemicals. Antioxidant supplements decrease oxidative DNA damage in humans (Duthie et al., 24), as do antioxidant-rich foods (Pool-Zobel et al., 25) Mitchell and Collins, 26 Collins et al., 27). The present investigated results are in agreement with that of Sram et al^[28] reported a high frequency of chromosomal aberrations in peripheral lymphocytes of coal-tar workers, occupationally exposed to polycyclic aromatic hydro carbons and benzene was reduced by ascorbic acid prophylaxis at a daily dose of 1 gr. Further, Sram et al reported a significant decrease in the frequency of chromosomal aberrations in peripheral blood lymphocytes of workers occupationally exposed to halogenated ethers. Furthermore, the present results are in augmentation with Pohl and Reidy^[29], where the intake of vitamin-C decreases the chromosomal damage in bleomycin drug induced in cancer patients. The inhibitory effects of ascorbic acid in various mammalian test systems were reported with insecticide Ragor^[30] Endosulphan, Phosphanidon and Mancozeb^[32] Cisplatin^[32] and Cyclophosphamide and Bleomycin.^[34] Further Cohen et al, (1993) reported that the chromosomal damage and DNA

stand breaks were reduced in mammalian cells in the presence of an antioxidant ascorbic acid.. A second relevant aspect of our results is the clear inhibitory effects of the genotoxicity of tobacco exposure by a continuous treatment with a complex polyvitamin mixture. These results agree with recent studies in Chernobyl clean-up workers (where the use of multivitamins as dietary supplement significantly decreases the frequency of chromosome aberrations, specially chromatid breaks; and confirming previous results where a decrease in occupational induced chromosome damage in lymphocytes after prophylaxis with vitamin C^[35] vitamins A and E, (Mierauskiene et al^[36], or polyvitamin complexes (Vaglenov et al.^[37] has been detected. Earlier we reported the protective effects of ascorbic acid in drugs and heavy metals induced cytotoxicity in *in vivo* and *in vitro* mammalian test systems.^[38- 43]

Vitamin C is a known free-radical scavenger and has been shown to inhibit lipid peroxidation in liver and brain tissue of lead-exposed animals. In lead-exposed rats, a minimal 500 mg/L concentration in drinking water was able to reduce ROS levels by 40 percent.^[44] In other animal studies, the toxic effects of lead on heme production were reversed by a vitamin C dose of 100 mg/kg.^[45] Other studies indicate vitamin C might have significant chelation capacity for lead. A human study, evaluating blood lead levels in pregnant women, found that 1,000 mg vitamin C per day, in addition to a prenatal multivitamin supplement, significantly lowered blood lead levels from a mean of 5.1 to 1.1 µg/dL during the course of pregnancy (West et al,^[46]) In a study of silver refining (involving lead smelting), workers with mean blood lead levels of 32.84 µg/dL and symptoms of lead toxicity (anemia, muscle wasting, abdominal colic) were given thiamine (vitamin B1) or vitamin C to evaluate the ability of these supplements to affect lead exposure.

In a study assessing the mechanism of vitamin C's lead-lowering capacity, 75 male smokers with no known occupational or residential lead exposure were given 1,000 mg vitamin C daily for 30 days.). Vitamin C, in combination with silymarin, has also been shown to effectively reduce the hepatotoxic effect of acute lead poisoning.^[47] The hypothesis that dietary antioxidant vitamins, minerals and trace elements play a significant role in reducing the incidence of human cancer, has received special interest during the last decades, but, although the overall results are promising, data seems insufficient to extract conclusive results.^[48]

However there are many results showing the antioxidant vitamin supplementation exhibits an overall protective effect against DNA damage induced in human cells by X-ray or H₂O₂

treatments, as demonstrated by using the comet test) or the micronucleus assay^[49] These results indicate that the effects of oxidative stress have the potential to be modified by the presence of antioxidants, the level of protection appear to depend on the nature and intensity of the oxidative stress. Supplementation of the chemo-preventative compounds has been known to be a strategy for protection against oxidative damage caused by environmental agents. The research development in this field has been established for the detection of anti-risk factors in human beings.

4. CONCLUSIONS

This work shows a clear genotoxic effect associated to the occupational exposure to high tobacco levels that can be significantly reduced by 3 month Ascorbic acid supplementation hence to the industry management it is advised to use the vitamin supplementation to the workers as the occupational exposure is genotoxic and the workers are continuously inhaled the fumes of tobacco dust has been proven as carcinogenic. The safety measures such as wearing gloves and masks and maintenance of the work place is very important for the health of the individuals. The results of our studies are useful for public health and medical community. Furthermore, increased micronuclei frequency in the grossly normal appearing oral mucosa of higher risk individuals is associated with greater risk of oral cancer development

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