Int J Hum Genet, 10(4): 239-245 (2010)
PRINT: ISSN 0972-3757 ONLINE: 2456-6360
DOI: 10.31901/24566330.2010/10.04.06

Cytome Assay of Buccal Epithelium for Bio-monitoring Genotoxic Assessment of Benzene Exposure among Petrol Pump Attendants

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KEYWORDS Buccal Cell. Micronuclei. *Trans. Trans*-Muconic Acid. Benzene. Phenol. Binucleats

ABSTRACT Petrol pump attendants are occupationally exposed to benzene through their contact with petrol vapor and engine exhaust. This study investigated the genotoxic effect associated with benzene exposure. Exfoliated buccal cells and urine samples were collected from 40 petrol pump attendants and 40 subjects as control group, their age and sex matched and they were not exposed to benzene. Further, these groups were classified into two subgroups: smokers and non- smokers. Cytogenetic study was carried out by cytome assay. To determine the benzene exposure, we have used metabolites of benzene such as phenol and trans, trans - muconic acid from urine. Frequencies of binucleated cells ($P < 0.01$), micronucleated cells ($P < 0.01$), bulging form nucleated cells ($P < 0.01$) and karyorrhectic cells ($P < 0.01$) were found to be significantly higher in petrol pump attendants than control individuals. Urinary mean phenol level ($P < 0.01$) and trans, trans - muconic acid levels ($P < 0.01$) were also found to be significantly high. Our study indicates that chronic and long term exposure of benzene can increase the genotoxic risk in petrol pump attendants.

INTRODUCTION

Benzene is a known carcinogen and evidences suggest that it could be associated with leukemia, so it is also known as leukemogen (Rinsky et al. 1981; Glass et al. 2001). Benzene is natural component of crude and refined petroleum. Benzene emissions to air are predominantly derived from road transport, mainly petrol. Benzene may also enter the environment through fugitive emission, from chemical manufacturing and processing operations and from refueling and distribution of fuels, principally petrol (EPA 1998; Glass et al. 2003).

The mandatory decrease of lead alkyls in gasoline has led to increase in aromatic hydrocarbon content of gasoline to maintain high octane levels and antiknock properties. In the United States, gasoline typically contains less than 2% benzene by volume but in other countries, the benzene concentration may be as high as 5%. But in India, petrol contains 3 - 5% of benzene by volume (ATSDR 2006; Glass et al. 2006).

Research has shown benzene to be a carcinogen. With exposure from less than 5 years to more than 30 years, individuals have developed and died from leukemia. Long exposure may in turn affect bone marrow and blood production (Rushton et al. 1997; Schnatter et al. 1996, 2005). Short term exposure to high levels of benzene can cause drowsiness, dizziness, unconsciousness and death. The current permissible exposure level is 1 part per million (ppm) in air for 8 hours average (EPA 1998).

Cigarette smoke is an important source of benzene in indoor air, and median benzene levels have been found to be higher in the homes of smokers (10.5 $\mu g/m^3$) than those of non-smokers (7 $\mu g/m^3$) in the USA (Hughes et al. 1994). Corresponding figures from Germany were 11 and 6.5 $\mu g/m^3$, respectively. The levels in the USA were higher than the corresponding median outdoor concentration, 6 $\mu g/m^3$, and the mean personal exposure was also higher at 15 $\mu g/m^3$. The mean concentration of benzene in indoor air in homes across Canada was 7.4 $\mu g/m^3$, with a maximum value of 68 $\mu g/m^3$. (WHO 2000; Fustinoni et al. 2005)

Inhaled benzene is absorbed by lung alveoli and mixes with blood, from where about 70% of

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benzene is directly exhaled by the lung into the air and remaining benzene is carried to the liver for the detoxification process of benzene and converts it into a number of metabolites like benzene - oxide, phenol, hydroquinone (HQ), and catechol. These metabolites, especially hydroquinone (HQ) are transported to bone marrow, the site of benzene toxicity, where they can be further metabolized through the action of peroxidase and converted into p benzo-quinone (p-BQ). The benzene metabolites HQ and p-BQ have been shown to cause a number of effects, both *in vivo* as well *in vitro*, which include the information of both DNA adducts and mutations (Kim et al. 2006). Cytochrome p450 opens benzene ring of benzene oxide (oxepin) and converts into non- cyclic compound trans, trans - muconic acid and aldehyde, where glutathione-*S*-transferase converts benzene oxide (oxepin) into *S*-phenylmercapturicacid. Phenol, trans, trans - muconic acid, and *S*-Phenylmercapturicacid are excreted in urine (Faiola et al. 2004; Rappaport et al. 2009). Therefore, the most common method for biological monitoring of benzene exposure is based on measuring a metabolite of benzene, usually urinary phenol. However, because of the high background of phenol, the result of its presence in many foods and of the metabolism of aromatic amino acids, measurement of phenol and phenolic metabolites such as hydroquinone and catechol is considered unreliable, especially for low amounts of benzene exposure (Ducos et al. 1990). So trans, trans-muconic acid (ttMA), a metabolite of trans, trans-mucoaldehyde through the oxidation of benzene oxide, has been also proposed as an indicator for monitoring benzene exposure in the study.

The oral epithelium maintains itself by a system of continuous cell renewal in which new cells produced by mitosis in basal layer migrate to the surface to replace those that are shed (Squier et al. 2001). Thus, the mucosa is composed of progenitor and maturing cell populations. Micronuclei are cytoplasmic chromatin masses with the appearance of small nuclei that arise from chromosome fragments or intact whole chromosomes lagging behind in the anaphase stage of cell division. Their presence in cells is a reflection of structural and/or numerical chromosomal aberrations arising during mitosis (Celik et al. 2003).

During a whole day shift, petrol pump attendants take low dose of benzene by inhaling volatile petrol fume containing benzene (Miller et al. 2005). In addition, cigarette smoke contains small amount of benzene, means who are cigarette smokers, get more benzene than the other non-smoker petrol pump exposed workers. Our purpose of study was to investigate genotoxic effect of benzene if any, on buccal epithelium of petrol pump workers by using cytome assay. Furthermore, we have investigated degenerative changes like binucleated cells, nuclear bud, karyolysis, and karyorrhectic cells.

MATERIALS AND METHODS

Study Population: The study was carried out from January 2009 to April 2009 on 40 petrol pump station attendants (20 smokers and 20 non-smokers), occupationally exposed to benzene. The control group consisted of 40 healthy men (20 smokers and 20 non-smokers) working in the University campus, without any indication of exposure to petroleum derivates or other potential genotoxic substances. Volunteer subject individuals were informed about the study design and asked to sign an informed consent and to fill in a questionnaire so as to obtain necessary data on their lifestyles and personal factors (age, working period, health, etc.). None of these study groups showed significant differences with regard to lifestyle and personal factors. All individuals were from Surat city.

Urine Collection: Urine samples were collected in white sterile plastic bottles from all subjects at the end of work shift. Urine samples (50 ml) were collected and preserved with 500 μl of 6 mol/l hydrochloric acid and stored in deep freezer.

Urinary Phenol Measurement: Urinary phenol measurements were performed following the colorimetric quantitative determination method of Yamaguchi and Hayashi (1977). The reported urinary phenol values were further corrected for creatinine content.

***Urinary Trans, Trans - Muconic Acid Measurement*:** After urine collection, within a week, urine samples were sent to the Centre for Salt and Marine Chemicals Research Institute, Bhavnagar, where for HPLC quantitative determination of trans, trans - Muconic acid was carried out by following method of Lee et al. (1993).

Evaluation of Micronuclei and Nuclear Abnormalities in Buccal Cells: After urine collection, subject individuals were asked to rinse

their mouths properly with water. Following which, buccal cell sampling was done. Disposable plastic spoon was used to obtain cell samples from buccal mucosa. The samples were then applied to clean microscope slides. Smears were air dried and fixed in methanol : acetic acid (3:1). Slides were stained by the 5% Geimsa. The slides were prepared in triplicate for each subject and 1000 cells were scored per slide to determine the micronucleus (MN) frequencies. Nuclear abnormalities were classified according to Tolbert et al. (1992). Briefly, cells with two nuclei were considered as binucleates. Nucleus with bulging form of nuclear material was of nuclear material was scored as nuclearbud. Nuclei fragmented into irregular pieces were scored as karyorrhexis. Nuclear dissolution, in which a Geimsa-negative, ghost-like image of the nucleus remains, was evaluated as karyolysis. The following criteria for micronucleus analysis were used in oral epithelial cells. A micronucleus must: (i) be less than one-third the diameter of the main nucleus; (ii) be on the same plane of focus; (iii) have the same color, texture and refraction as the main nucleus; (iv) have a smooth, oval or round shape; (v) be clearly separated from the main nucleus.

Statistical Analysis: Data were pooled as the mean ± standard error of the mean. Data were tested for normality by the Kolmogorov-Smirnov test. Statistical analyses of data were performed using two tailed Student's "t"-test assuming unequal variance. The type I error rate (α) was accepted as 0.05. All statistical analyses were performed using the program Microsoft Excel 2007 and verified by SPSS 15 for the PC.

RESULTS

All data including urinary phenol and trans, trans - muconic acid, micronuclei were found normal by the Kolmogorov-Smirnov test. Table 1 shows demographic characteristic of Urinary phenol and trans, trans - muconic acid data with age and duration of employment, whereas table 2 shows summary of cytome assay parameters.

Mean urinary phenol value in petrol pump attendant (66.01 ± 2.60 mg/g/l creatinine) was found to be significantly higher ($P < 0.01$) than control individuals (43.98 ± 2.32 mg/g/l creatinine). Likewise, mean urinary phenol in smoker petrol pump attendants were found significantly higher ($P < 0.05$) than mean urinary phenol content of smoker control individuals.

Table 1: General characteristics of the groups studied

Study group		*N*	*Age (years)*	*Mean duration of employment (years)*	*Urinary phenol (mg/g/l creatinine)*		*Urinary trans, trans-muconic acid(mg/g/l creatinine)*	
			Mean ± SE		*Range*	*Mean ± SE*	*Range*	*Mean ± SE*
Control individuals	Smokers	20	31.00 ± 1.76		23 - 71	60.80 ± 3.41	0.17 - 0.56	0.45 ± 0.05
(Non exposed to benzene)	Non smokers	20	28.80 ± 2.70		13 - 54	16.16 ± 2.80	0.09 - 0.32	0.14 ± 0.07
	Total	40	29.84 ± 1.62		13 - 71	43.98 ± 2.32	0.09 - 0.56	0.29 ± 0.06
Petrol pump workers	Smokers	20	31.20 ± 0.90	8.28 ± 0.82	45 - 135	91.44 ± 5.79	0.54 - 3.14	1.48 ± 0.13
(Exposed to benzene)	Non smokers	20	27.30 ± 1.21	6.95 ± 0.83	30 - 132	50.58 ± 5.84*	0.12 - 1.02	0.31 ± 0.03*
	Total	40	29.24 ± 0.77	7.61 ± 0.59	30 - 135	66.01 ± 2.60**	0.12 - 3.14	0.90 ± 0.08**

Significant difference from control at *($P<0.05$) and **($P<0.01$).

Whereas, mean urinary phenol value in non-smoker petrol pump attendants were also found significantly higher ($P < 0.05$) than non- smoker control individuals.

Urinary trans, trans - muconic acid mean value in petrol pump attendants (0.9 ± 0.08 mg/g/l creatinine) was found to be significantly higher ($P < 0.01$) than control individuals (0.29 ± 0.06 mg/g/l creatinine). Comparison of petrol pump attendant smokers with control smoker individuals were seen significantly higher ($P < 0.05$).

Assessment of micronuclei frequencies in exfoliated buccal cells revealed a significant difference ($P < 0.01$) between petrol pump attendants (11.56 ± 0.43) and control individuals (6.00 ± 0.25) and smoking also had a significant effect on micronuclei frequency in both exposed and control groups ($P < 0.01$).

Binucleus frequency was observed significant between control individuals (6.19 ± 0.27) and exposed (17.71 ± 0.57) workers ($P < 0.01$). Nuclear bud frequencies also observed significantly higher in petrol pump attendants (6.26 ± 0.27) than control individuals (3.09 ± 0.19).

Karyorrhexis frequency was found to be higher ($P < 0.01$) in petrol pump attendants (13.79 ± 0.49) than control individuals (4.38 ± 0.24). Similarly, karyolysis frequency was found to be significantly higher ($P < 0.01$) in petrol pump attendants (17.29 ± 0.55) than in the control individuals (6.11 ± 0.32).

Table 2: The frequencies of micronuclei and other nuclear abnormalities in exfoliated buccal cells of controls and exposed subjects

Study group		*n*	*Nuclear abnormality per 1000 cells*				
			Micronuclei	*Binucleates*	*Nuclear bud*	*Karyorrhexis*	*Karyolysis*
Control Individuals	Smokers	20	7.18 ± 0.32	7.28 ± 0.40	4.15 ± 0.24	5.85 ± 0.27	7.96 ± 0.37
	Non Smokers	20	4.80 ± 0.27	5.10 ± 0.27	2.03 ± 0.18	2.90 ± 0.21	4.25 ± 0.32
	Total	40	6.00 ± 0.25	6.19 ± 0.27	3.09 ± 0.19	4.38 ± 0.24	6.11 ± 0.32
Petrol Pump Workers	Smokers	20	13.10 ± 0.67*	20.30 ± 0.80*	7.50 ± 0.36*	15.88 ± 0.73*	20.90 ± 0.60*
	Non Smokers	20	10.08 ± 0.43*	15.03 ± 0.54*	5.03 ± 0.31*	11.70 ± 0.47*	13.68 ± 0.43*
	Total	40	11.56 ± 0.43*	17.71 ± 0.57*	6.26 ± 0.27*	13.79 ± 0.49*	17.29 ± 0.55*

Significantly difference from control at *($P<0.01$).

DISCUSSION

Benzene is a natural component of crude oil, and petrol. Production of petrol 1988 was estimated to be 20 million tones worldwide and 5 million tones within the countries of the European Economic Community. Occupational exposure to benzene is experienced during its production and from coke ovens. Besides these industrial sources, emission also occurs from different combustion sources, such as motor engines, wood combustion and stationary fossil fuel combustion. The major source is exhaust emissions and evaporation losses from motor vehicles, and evaporation losses during the handling, distribution and storage of petrol (WHO 2000).

Aksoy et al. (1985) and Hayes et al. (2000) concluded that occupational exposure to benzene has mainly been associated with

increased incidences of acute myeloid leukemias. Additionally, Wong et al. (2000), Goldstein et al. (2000) and Miller et al. (2005) have reported incidences of chronic myeloid and acute lymphoid leukemia, multiple myeloma and non-Hodgkin's lymphomas respectively, among individuals exposed to benzene. Kesavachandran et al. (2006) observed some lung function abnormities in benzene exposed individuals. In addition, Zhang et al. (1998, 2005) and Smith et al. (2000) observed that occupational benzene exposure may increase aneusomy and long arm deletion of chromosome no. 5 and 7. Earlier epidemiological studies have shown a clear relationship between the increase in MN frequency and exposure to benzene and/or benzene metabolities (Yager et al. 1990; Tompa et al. 1994; Turkel and Egeli 1994).

Nevertheless, available data on cytogenetic damage in petrol station attendants are contradictory. Evaluation of SCE frequencies in petrol station attendants showed that there was no significant difference between controls and experimental subjects (Carere et al. 1995; Pitarque et al. 1997). Bukvic et al. (1998) also analyzed MN (micronucleus) and SCE (sister chromatid exchange) frequencies in peripheral lymphocytes of petrol station attendants and failed to reveal any significant differences for SCE, unlike significant differences for MN.

In the present study, we observed increased in micronucleated buccal cells frequency in petrol station workers than control individuals. Our findings are consistent with the results of Hogstedt et al. (1991), who detected a significant increase in the frequency of micronuclei in the lymphocytes of petrol station workers. Celik et al. (2003) also observed increased frequency of micronucleus from buccal mucosa cells of petrol pump attendants of Mersin city, Turkey.

Cytome assay of exfoliated cells of buccal mucosa provides evidence of other nuclear abnormalities such as binucleates, karyorrhexis, nuclear bud and karyolysis (Fenech et al.1999; Thomas et al. 2007). Binucleus formation is considered as indicator of cytotoxicity, while karyorrhexis and karyolysis are considered as indicators of apoptosis. In our study, we found significant increased nuclear abnormalities in petrol pump station attendants. Our findings are in agreement with those of Celik et al. (2003), who detected a significant increase in the frequency of nuclear abnormalities in the buccal cells of petrol station workers than the control individuals. Our findings indicate that cigarette smoking has significant effected in increasing the frequencies of MN and other nuclear abnormalities in both petrol pump attendants and control individuals.

Benzene metabolites are useful to measure the low dose benzene exposure and hence are good biomarkers. Phenol is the principal metabolite of benzene. Gasoline or benzene exposed individuals revealed increased urinary phenol level than controls (Hein et al. 1989; Verma and Rana 2001). A study on Indian traffic policemen occupationally exposed to vehicular smoke containing benzene showed increased levels of urinary phenol (Verma et al. 2003). Khoschsorur and Peter (2000) reported that urinary phenol concentration in petrol station workers was higher than the control subjects. In our study, we also found significantly higher urinary phenol levels in petrol station workers than in the control individuals.

Nevertheless, the urinary phenol levels can be affected by demographic factors, such as diet, smoking and ingestion of medicine (Bechtold et al. 1991). Therefore, we have analyzed other urinary biomarker named trans, trans-muconic acid for biomonitoring of low level benzene exposure (Lee et al.1993; Qu et al. 2000). Normal population who are not exposed to benzene also contain very low concentration of trans, trans-muconic acid. Whereas smokers who were exposed to low dose of benzene showed significantly increased trans, trans-muconic acid level in urine (Lee et al.1993). In our study, we observed significantly increased levels of trans, trans-muconic acid in petrol pump station attendants than control individuals. Even smokers had increased trans, trans-muconic acid levels in both petrol pump attendants and control individuals.

In conclusion, data presented here support that petrol pump attendants are under risk of cytogenetic damage and are more vulnerable to malignancy or cancer. Additionally, cytome assay and urinary trans, trans - muconic acid levels are a very good tool to measure genotoxic effect of benzene. However, more studies are required for a meaningful conclusion.

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