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Cytogenetic Damage in Offset Printing Press Workers Occasionally Exposed to Benzene

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INTRODUCTION

According to W.H.O. (1993) Benzene is a stable colourless liquid at room temperature and normal atmospheric pressure. It has a characteristic aromatic odour, a relatively low boiling point (81.1°C) and a high vapour pressure which causes it to evaporate rapidly at room tempera-ture, and is highly flammable. It is slightly soluble in water but miscible with most other organic solvents.

Benzene is a naturally occurring chemical found in crude petroleum at levels upto 4g/liter. It is also produced in extremely large quantities (14.8 million tonnes) worldwide. Emissions arise during the processing of petroleum products, in the coking of coal, during the production of toluene, xylene and other aromatic compounds, and from its use in consumer products, as a chemical intermediate and as a component of gasoline (petrol).

Benzene in air exists predominantly in the vapour phase, with residence times varying between a few hours to a few days, depending on environment and climate, and on the concentration of hydroxyl radicals, as well as nitrogen and sulfur dioxides. The presence of benzene in gasoline (petrol), and as a widely used industrial solvent can result in significant and widespread emissions to the environment. Out door environmental levels range from 0.2 mg/m^3 in remote rural areas to 3.49 mg/m^3 in industrial centres with high density of automobile traffic. During refuelling of automobiles, levels up to 10 mg/m^3 have been measured.

Benzene has been detected at levels as high as 500 mg/m^3 in indoor residential air. Cigarette smoke contributes significant amount of benzene to the levels reported in indoor air, with smokers inhaling approximately 1800mg benzene/day compared to 50 mg/day by non-smokers. It is known that benzene produces a number of adverse health effects. The most frequently reported health effect of benzene is bone marrow depression leading to aplastic anaemia. At high levels of exposure a high incidence of these diseases is probable.

Benzene is a well established human carcinogen. Epidemiological studies of benzene-exposed workers have demonstrated a casual relationship between benzene exposure and the production of myelogenous leukemia. Benzene has given negative results in mutagenicity assays *in vitro*.

In *in vivo* studies, benzene or its metabolites cause both structural and numerical chromosome aberrations in human and laboratory animals. In addition, benzene administration results in the production of sister chromatid exchanges and polychromatic erythrocytes with micronuclei. Benzene can reach germ cells after intra peritoneal dosing, as shown by the production of abnormalities in sperm head morphology.

The adverse effects of an exposure to various chemicals have gained considerable attention of the scientific community. The use of genotoxicity is essential for assessment of potential human toxicity so that hazards can be prevented. These days printing has become a very important occupation. By this process the image is transferred from the metal plate to a substrate. Commercial printers mostly use offset, thermography, flexography and rotogravure techniques. In the present study offset printing press workers were investigated for exposure to benzene and its derivatives, which are important substances widely used in industry as solvents, as fuel and in the manufacture of drugs and dyes. In offset printing machine benzene is used for getting the pressed rubber blanket swollen. The workers who use benzene in a printing press are the machine man and the inker. Chronic benzene intoxication modulates immune response, granulocyte enzyme systems, leads to thrombocytopenia, anemia and even pancytopenia. In extreme cases it leads to neoplastic diseases (Cronkite, 1987; Dean, 1978; Moszczynski and Lisiwicz, 1983; Smolik et al., 1973). The frequency of neoplasia in workers professionally exposed to benzene and its derivatives is significantly higher than in the unexposed ones (HoneyCombe, 1978; Rinsky et al., 1987) Occupational exposure to benzene has also been associated with various blood dyscrasias and increased incidence of acute myelogenous leukemia in man (Subrahmanyam et al., 1990). Cytogenetic analysis of human

chromosomes in peripheral lymphocytes allows direct detection of mutations in somatic cells. As such the present in vitro cytogenetic study was undertaken on 65 workers exposed to benzene in off set printing press and similar number of unexposed controls matched with respect to age, sex, smoking and drinking habits to investigate the genotoxicity of benzene in them.

MATERIAL AND METHODS

This study comprised 65 workers exposed to benzene in printing press and similar number of unexposed controls matched with respect to age, sex, smoking and alcohol habits.

An epidemiological survey was conducted using a proforma specially designed for this purpose.

Blood samples were taken using heparinized syringes. Short term lymphocyte cultures were set up within 4h of sampling following the technique of Moorhead et al. (1960) with minor modifications.

Lymphocytes were cultured by adding 0.5 ml of blood in 5 ml of RMPI 1640 medium (Hi Media) supplemented with 20% foetal calf serum and 0.1 ml of phytohaemagglutinin (Sigma). Colchicine 10 µg/ml (sigma) was added to the culture prior to harvesting.

Lymphocytes were harvested after 48h for CA. Slides were prepared and stained with 4% Giemsa solution (E. Merck). For each person 100 well spread metaphases were analysed.

For sister chromatid exchanges (SCE) 5-bromo-deoxyuridine (Brdu, 10 ug/ml culture) was added 24h after setting up of the cultures. Harvesting was done after 72 h. Slides were prepared by air drying method, and stained with Hoechst 33258 (Sigma) and 4% Giemsa solution following the method of Perry and Wolf (1974). For calculating the frequency of SCE per cell, 25 metaphases were analysed as per international norms.

For evaluating the frequency of satellite associations (SA), 100 good metaphases were scanned. The criteria described by Hansson (1970) were followed for evaluating the SA. The criteria are (i) The satellite ends of the associating chromosomes had to be directed towards each other with their longitudinal axes meeting between their short arms.

(2) The distance between the centromeres of associated chromosomes should not exceed the total length of one G chromosomes, its satellite excluded.

The samples were analyzed under code by two observers, such that the observers were unaware of the identity of the sample, in order to remove possible laboratory scoring bias.

For statistical analysis of results Student's t-test was applied.

RESULTS

Epidemiological study showed that most of the exposed workers complained of restlessness, muscular twitching, headache and nausea.

The data on various parameters obtained during the present investigations have been given in tables 1-6.

The mean percentage of the CA found in the exposed group was (2.78) and that in the controls was (0.88) resulting in highly significant difference at ($p<0.01$) (Table 1). Both chromosome type aberrations viz. dicentrics, rings, acentric fragments, translocations, chromosome gaps and chromosome breaks and chromatid type aberrations viz. chromatid gaps and chromatid breaks were encountered in the exposed group. All the values for these aberrations were significant at $P<0.01$. In the control group rings, translocations and chromosome breaks were not observed. There was a significant difference in the frequency of CA among smokers and alcoholics

Table 1: Frequency of chromosomal aberrations in workers exposed to benzene in offset printing press and control individuals

Group	*Control individuals*	*Exposed individuals*
No. of Individuals	65	65
No. of Metaphases	6500	6500
Chromosome Type Aberrations		
Dicentrics	2 (0.03)	5(0.07)
Rings	-	3 (0.04)
Acentric Fragments	3 (0.04)	10 (0.15)
Translocations	-	2 (0.03)
Chromosome Gaps	4 (0.06)	13 (0.20)
Chromosome Breaks	-	6 (0.09)
Total (without gaps)	5 (0.07)	26 (0.40)
Chromatid Type Aberrations		
Gaps	88(1.35)	183(2.81)
Breaks	52(0.80)	155(2.38)
Isochromatid Exchanges	0	0
Total (without gaps)	52(0.80)	155(2.38)
Total Chromsomal Aberrations (without gaps)	57(0.88)	181(2.78)*

Values in parantheses indicate aberrations per 100 metaphases.
* Significant at $P<0.01$

as compared with non smokers (1.37). Exposed alcoholic smokers showed a higher value (4.61) as compared with exposed non alcoholic smokers (2.05) (Table 2).

Table 2: Frequency of chromosomal aberrations (CA) in offset printing press workers and control individuals

Group	*Control individuals*		*Exposed individuals*	
	n	*Mean ± S.D.*	*n*	*Mean ± S.D.*
Total Individuals	65	0.87 ± 0.89	65	2.78 ± 1.90*
Smokers	29	1.37 ± 0.97	29	4.24 ± 1.84
Non-Smokers	36	0.47 ± 0.55	36	1.69 ± 0.98
Alcholics	32	1.21 ± 1.00	30	3.73 ± 2.03
Non-Alcholics	33	0.54 ± 0.61	35	2.05 ± 1.41
Smoker Alcholics	19	1.57 ± 0.96	18	4.61 ± 2.03

* Significant at P < 0.01
n - Number of Samples

Table 3 shows the frequency of SCE in exposed workers and their controls. A significant difference (P<0.01) was observed in the induction of SCE in exposed workers (9.08) as compared with controls (3.72). The smokers, both exposed (9.56) and control group (3.87), showed higher values of SCE. The frequency of exposed alcoholics (9.47) was higher as compared with exposed non alcoholics (8.75). The value of SCE in the alcoholic smokers is 9.62 which is more than exposed smokers, non-smoker alcoholics; and non-alcoholics (Table 4).

Table 3: Frequency of CA and SCE in printing press workers with duration of exposure

Duration of exposure (In years)	*Number of individuals*	*CA* *Mean ± SD*	*SCE* *Mean ± SD*
0-5	20	1.00 ± 0.64	8.61 ± 0.44
6-10	18	2.44 ± 1.04	9.11 ± 0.64
11-15	16	3.87 ± 1.62	9.27 ± 0.56
16-20	11	5.27 ± 1.19	9.67 ± 0.60

Table 4: Frequency of sister chromatid exchanges (sce) in exposed and control individuals

Group	*Control individuals*		*Exposed individuals*	
	n	*Mean ± S.D.*	*n*	*Mean ± S.D.*
Total Individuals	65	3.72 ± 0.21	65	9.08 ± 0.65*
Smokers	29	3.87 ± 0.21	29	9.56 ± 0.45
Non-Smokers	36	3.68 ± 0.16	36	8.68 ± 0.50
Alcholics	32	3.83 ± 0.21	30	9.47 ± 0.52
Non-Alcholics	33	3.63 ± 0.17	35	8.75 ± 0.56
Smoker Alcholics	19	3.97 ± 0.14	18	9.62 ± 0.50

Table 5 shows the frequency of SA in exposed and control individuals. The types of SA observed were DD, DG, GG, 2DG, 2GD, 2D2G and 3D. The SA showed highly significant values (P<0.01) in the exposed samples (11.75 ± 6.34) as compared with the controls (4.53 ± 3.27). DG type associations were maximum, while 3D type were minimum.

Table 5: Frequency of satellite associations observed in exposed and control individuals

Type of Satellite associations	*Number of Individuals* *Control individuals*	*Exposed individuals*
	65	65
Total Cells Scanned	6500	6500
Type of Satellite Associations		
DD	57 (0.87)	147 (2.26)
DG	150 (2.30)	332 (5.10)
GG	41 (0.63)	139 (2.13)
2DG	19 (0.29)	58 (0.89)
2GD	10 (0.15)	42 (0.64)
2G2D	11 (0.16)	26 (0.40)
3D	7 (0.10)	20 (0.30)
Total	295 (4.53)	764 (11.75)
Associations Per Cell	4.53 ± 3.27	11.75 ± 6.34*

Figures in parentheses indicate the satellite associations per 100 metaphases
*Significant at P <0.01

Table 6 shows the frequency of MN in exposed and control individuals. The MN showed highly significant values (P<0.01) in the exposed (0.57) as compared with the control individuals (0.45).

Table 6: Frequency of micronuclei (mn) in offset printing press workers and control individuals

Sample	*No. of samples (n)*	*MN* *Mean ± S.D.*
Control	25	0.45 ± 0.11
Exposed	25	0.57 ± 0.11*

*Significant at P<0.01

DISCUSSION

In the present study most of the exposed workers complained of muscle twitching, restlessness, headache and nausea. Earlier Bensley and Joron (1963) reported that benzene acts as a local irritant. Chronic poisoning is an important industrial hazard but acute poisoning is uncommon.

Extensive *in vitro* genotoxicity testing has indicated that benzene is weekly mutagenic or non-mutagenic in standard mutation assays but

does cause chromosomal aberrations (IARC, 1982; Dean, 1985; Waters et al., 1988).

During the present investigation workers exposed to benzene showed significantly increased CA as compared to the matched controls. The gaps were not taken into consideration because their significance in cytological monitoring of populations is still a matter of discussion (Brogger, 1982). However, among exposed as well as control group the total chromatid type aberrations were far more than the total chromosome type aberrations.

Cytogenetic studies in benzene-exposed workers have reported a similar profile of genotoxicity. Increased frequencies of structural CA in the lymphocytes of benzene-exposed workers have been reported by numerous investigators (Sorsa and Yager, 1987; Aksoy, 1988). Elevated frequencies of aneuploid cells have also been observed in the lymphocytes of workers with occupational exposure to benzene (Forni et al., 1971; Haberlandt and Mente, 1971; Ding et al., 1983).

Picciano (1979) conducted cytogenetic analysis on 52 workers exposed to less than 10 ppm and 44 matched controls he found a small, but statistically significant increase in CA. In a later analysis of the data he reported a dose related increase in CA when the exposed workers were divided into small groups by exposure. Dean (1985) criticised his findings on several logical accounts and concluded that chronic benzene exposure below 10 ppm induces chromosome aberration must be treated with caution. A similar conclusion must be drawn from the studies of Fredga et al. (1979).

According to Neumeier (1992) benzene consistently induces chromosome aberrations in human bone marrow cells and peripheral lymphocyte at long term exposure level > 25 ppm. (80 mg/m^3). Major et al. (1994) provide evidence that chromosomal aberrations of peripheral lymphocytes may occur at benzene exposure level < 10 ppm. (32 mg/m^3). Previous studies suggested that when the exposure is well below the occupational exposure limit for benzene among other genotoxic chemicals, their is no biologically significant increase in the frequencies of CA in lymphocytes of workers (deJong et al., 1988).

Sasiadek et al. (1989) found significantly increased frequency of metaphase cells with structural chromosomal aberrations over that observed in a control population (47% vs 1.7%) in workers occupationally exposed to benzene and its derivatives. A statistical analysis of chromosomal distribution of the break points involved in the aberration indicated that while in control individuals the distribution was random, it was non-random in the cells of individuals exposed to benzene. Smith et al. (1998) have shown that benzene exposure is associated with markedly elevated levels of t(8;21) and of hyper diploidy of chromosomes 8 and 21, in the circulating lymphocytes of otherwise healthy workers exposed to benzene compared to unexposed controls. This suggests a role for these aberrations in benzene-induced leukemia and that the detection in peripheral blood cells by chromosomal painting may be useful biomarkers of increased risk for haematological malignancies for benzene and other potential leukemogens.

Zhang et al. (1998a) reported that benzene exposure was associated with increase in the rates of monosomy 5 and 7 but not monosomy 1 ($P<0.001$, $P<0.0001$ and $P = 0.94$ respectively) and with increase in trisomy and tetrasomy of all three chromosomes long arm deletion of chromosomes 5 and 7 was increased in dose dependent factor ($P<0.014$ and $P<0.0001$) up to 3.5-fold in the exposed workers and suggested that aberrations in chromosomes 5 and 7 may be useful biomarkers of early biological effect for benzene exposure. Zhang et al. (1998b) also reported the loss and long arm deletion of chromosomes 5 and 7 in human lymphocytes induced by benzene metabolites.

Significantly higher values of SCE as compared with respective controls have been observed in the study. The background level of SCE was 3.72 per cell. Morimoto et al. (1983), Sarto et al. (1984) Tice et al. (1979, 1981) and Funes-Cravioto et al. (1977) observed an increase in the frequency of SCE due to benzene. But Gerner Smidt and Friedrich (1978) did not observe any effect of benzene and toluene on the frequency of SCE.

Increased frequency of SCE and CA with the increasing duration of exposure have been observed in the present study. Similar results were shown by Major et al. (1994).

The effect of long term benzene exposure on the incidence of SCE and structural aberrations was investigated by Watanbe et al. (1980) who did not find any increase in CA though the incidence of SCE appeared to be less than that in controls. Dean (1985), taking into account the small sample of workers and the small reduction of SCE, has cautioned that this observation and its interpretation must be regarded as speculative. Sarto et al. (1984) observed differences in SCE between exposed and matched control

workers. He further compared 'high' exposure and 'low' expsoure workers which suggested a correlation between level of exposure to benzene and reduction in the incidence of SCE confirming the observation of Watanbe et al. (1980). A small increase in chromosome type of aberration was seen in the benzene workers though their was no significant effect on the frequency of chromatid type aberration. Clare et al. (1984) analysed chromosomes of peripheral blood lymphocyte from a group of workers after a single high exposure to benzene conducted about after three months of exposure. There was no significant difference in the incidence of aberration between the exposed workers and control group, although some of the exposed workers showed a slightly higher number of SCE than control group. It was concluded that there was no evidence of lasting chromosomal damage in the peripheral blood lymphocytes of the exposed workers.

The micronucleus assay is a simple and rapid technique which permits the identification of agents which cause both structural and numerical aberrations.

In the present investigation the increased MN were observed in the exposed workers as compared to controls. Similar results were earlier shown by Nise et al. (1991) and Tice et al. (1984). Luke et al. (1985) found the increased effect of benzene with the increased exposure time.

Yager et al. (1990) investigated ability of principal phenolic and quinonoid metabolites of benzene to induce structural numerical chromosomal aberrations by micronucleus assay in treated human peripheral lymphocytes and found that all of the tested benzene metabolites induced significant dose-related increase in micronuclei.

During the present investigation increased SA were observed in workers exposed to benzene as compared to controls. Similar results were earlier reported by Rita et al. (1987) in grape garden workers occupationally exposed to different pesticides, by Yadav and Kaushik (1996, 1997) in fertilizer factory workers exposed to SO_2 and NH_3 gases respectively and by Yadav and Seth (1998 a,b) in NO_x and PAH exposures Yadav and Thakur (2000 a,b) in tobacco smokers. The significant increase in various parameters during present investigation, thus reveal the genotoxic potential of benzene on human peripheral lymphocytes.

The genotoxicity of benzene seems to be mediated by multiple metabolites acting on multiple sites and in a synergistic fashion (Chen and Eastmond, 1995). Benzene can be metabolized to give ring hydroxylated and ring open metabolites with genotoxic properties (Snyder et al., 1993).

Activation of phenolic metabolites of benzene produces active oxygen which is capable of causing oxidative DNA damage which may play a role in benzene-induced genotoxicity, myelotoxicity and leukemia (Kolachana et al., 1993). Simultaneous exposure of human lymphocytes to two benzene metabolites CAT and HQ, resulted in striking synergistic induction of MN in human lymphocytes (Robertson et al.,1991). This provides considerable support to the growing acceptance that benzene-induced myelotoxicity and genotoxicity is not the effect of single metabolite acting at a single target site (Tice et al.,1982; Goldstein, 1989; Snyder et al.,1989; Barale et al.,1990), but rather multiple metabolites acting at several target sites, all of which may be critical to the onset of neoplasia.

Bearing in mind the leukemogenic potential of benzene peripheral blood lymphocytes can be considered a cell system relevant for biomonitoring benzene exposed workers (Surralles et al., 1997). Recent studies have suggested that high frequency of chromosomal aberrations in peripheral lymphocytes are predictive of increased cancer risk in humans (Hagmar et al., 1994; Bonassi et al. 1995).

With the development of new techniques in molecular biology the way is probably open to investigate some of the outstanding problems in research into benzene toxicity. Identification of the critical adducts in the DNA and other macromolecules should be possible with the further refinement of monoclonal methods and should lead clearer definition of the electrophiles responsible for the genotoxic effect of benzene.

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KEY WORDS Benzene. Peripheral Blood Lymphocytes. Chromosomal Aberrations. Sister Chromatid Exchanges. Satellite Association. Micronuclei.

ABSTRACT Cytogenetic investigations on peripheral blood lymphocytes of 65 workers exposed to Benzene in offset printing press were undertaken. These were compared with an equal number of occupationally

unexposed and matched controls in relation to age, sex, smoking and drinking habits. The chromosomal aberrations (CA), sister chromatid exchanges (SCE), satellite associations and micronuclei (MN), were analysed. All the parameters showed a significant increase ($P<0.01$) in the exposed sample compared with the controls viz CA, 0.88-2.78, SCE, 3.72-9.08, SA, 4.53-11.75 and MN, 0.45-0.57. The occurence of DG type satellite association was highest and that of 3 D type lowest

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