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Estimation of cytogenetic risk among coke oven workers exposed to polycyclic aromatic hydrocarbons

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Polycyclic aromatic hydrocarbons (PAHs) result from the incomplete combustion of natural or synthetic organic materials. The working environment at a coke plant can negatively affect the employed workers who were exposed to coke oven emissions containing PAHs, which formed and released into the environment by the process of pyrolysis of coke. This study aims to analyze the relationship between the exposure of PAHs and the risk of genetic damages such as chromosomal alteration (CA), micronucleus (MN), and DNA damage (PCR-RFLP) in peripheral blood lymphocytes of 27 coke oven workers and equal number of control subjects. The exposed subjects and controls were divided into two groups based on their age (group I<35 years and group II ≥35 years). The exposed subjects were further classified into two groups based on the exposure period (<12 years and ≥12 years). The frequencies of CA and MN in exposed subjects are relatively high with respect to controls. The XRCC1 399 Arg/gln polymorphism showed a substantial smaller difference in allele frequencies between exposed and control subjects. Based on present data, it was concluded that coke oven workers under risk should be monitored for adverse effects of the any long-term exposure.

Key words: polycyclic aromatic hydrocarbons (PAHs), coke oven workers, chromosomal aberrations (CA), micronucleus (MN), XRCC1

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INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are well known, widely distributed carcinogenic compounds. Coke oven workers are frequently exposed to high concentrations of PAHs (Wu *et al.,* 1998; Chen *et al.,* 1999; Kuljukka-Rabb *et al.,* 2002; IARC 2010), especially those working at the top sides of the ovens. An incomplete combustion of organic compounds in the environment was identified as a major source of carcinogenic risk. It constitutes a major part of air pollution in the coke oven area (IARC, 1987). PAHs have been identified as cancer-inducing chemicals for humans (IARC, 1983; Yang *et al*, 2002) and the exposure to those compounds at the workplace has been analyzed by the increased levels of biomarker, such as peripheral blood lymphocyte culture (Hemminki *et al.,* 1990; Bender *et al.,* 1992; Clonfero *et al.,* 1995). Epidemiological studies have associated a higher incidence of lung, scrotal, bladder, and skin cancer with occupational PAHs exposure (Dipple *et al.,* 1984; IARC, 1984; Armstrong *et al.,* 2004; Marczynski *et al.,* 2005; Chen *et al.,* 2007). Complex interactions between environmental and genetic factors are involved in the development of most of the human cancers (Perera, 1997). Besides bulky DNA adducts of carcinogenic PAHs, such as Benzo(a)pyrene diolepoxide (BPDE) — DNA adduct, a wide variety of non-bulky base damage and single-strand breaks formed by free radicals during metabolic activation of PAHs are also involved in the PAH carcinogenesis (Frenkel, 1992; Bankson *et al.,* 1993; Pryor, 1997). Consequently, genetic variation of an individual's ability to repair DNA base damage and single-strand breaks may confer differential risk for PAH-induced lung cancer. In humans, the base excision repair (BER) is responsible for the repair of oxidative DNA damage (Hoeijmakers, 2001). Since the chromosomal damage has been recognized as an important early biological event in chemical carcinogenesis, the relationship between genetic polymorphisms of BER proteins and PAH-induced chromosomal damages is important for the understanding of PAH carcinogenesis.

The cytogenetic analysis of peripheral lymphocytes is a method traditionally used to evaluate occupational exposure to clastogens (Hungerford, 1965; Carrano & Natarajan, 1982, Kalina *et al.,* 1991). The study of cytogenetic endpoints in lymphocytes, such as structural chromosomal alteration (CA) and micronucleus (MN), which are biomarkers of early biological effects of exposure to genotoxic agents, is considered advisable in transitional studies of groups at risk of occupational cancer, together with the determination of indicators of exposure and of biologically effective doses (Aitio *et al.,* 1988). The XRCC1 gene participates in the repair of mammalian DNA and plays an integral role in BER (Wood *et al.,* 2001). A polymorphism at codon 399 of the XRCC1 gene results in an amino acid substitution from arginine to glutamine in the BRCA1 C terminus domain of the gene, a region required for the efficient

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Abbreviations: BER, Base Excision Repair; BPDE, benzo(a)pyrene diolepoxide; CA, chromosomal alteration; MN, micronucleus; PBLC, peripheral blood lymphocytes culture; OSHA, Occupational Safety and Health Administration; PAHs, polycyclic aromatic hydrocarbons.

repair of single strand-breaks (Taylor *et al.,* 2002). The functional significance of the XRCC1 Arg399Gln polymorphism has been evaluated in several studies (Ginsberg *et al.,* 2011; Taylor *et al.,* 2002; Matullo *et al.,* 2001; Duell *et al.,* 2000; Abdel-Rahman & El-Zein, 2000; Lunn *et al.,* 1999). The Gln allele variant was associated with an increased level of sister chromatid exchanges (SCEs) after exposure to the tobacco-specific nitrosamine, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK) (Abdel-Rahman & El-Zein, 2000). In addition, a higher level of SCEs and polyphenol DNA adducts were present in smokers' lymphocytes, homozygous for the 399 Gln allele (Duell *et al.,* 2000). The present study aims to investigate the relationship between the exposure of PAHs and the risk of genetic damages, such as CA and MN in peripheral blood lymphocytes (PBLC) and gene polymorphism among smoking and non-smoking coke oven workers.

MATERIALS AND METHODS

Study area and subject recruitment. The study was carried out in 27 coke oven workers from a steel company in Coimbatore district, South India. The control group consisted of 27 healthy subjects selected based on the questionnaire that covered standard demographic questions (age, genetic disorders, number of X-ray diagnosis, vaccinations, medication, smoking, alcohol, etc.). The control group included healthy individuals selected from the same area, but who were not exposed to PAHs or any other chemicals and/or radiations. In our study, all the exposed subjects were selected from the top side, middle side, and bottom working areas of the coke oven plant. In addition, higher permissible exposure limit of PAHs was measured at the workplace which exceeds the exposure limit mentioned by the Occupational Safety and Health Administration (OSHA). The coke oven gas consists of 2% of CO_2, 5.5% of CO, 32% of CH_4, 51.9% of H_2, 0.3% of O_2, and 4.8% of N_2. In our study the exposed subjects between 20 to 53 years of age employed in coke oven plant were recruited. The employment duration ranges from 0.5–13 years; therefore, the groups were categorized based on the average age and exposure duration as group I (< 35 yrs of age and < 12 yrs of exposure) and group II (≥35 yrs of age and ≥12 yrs of exposure), respectively.

Sample Collection. At the time of blood collection (3 ml/individual) the workers signed a term of informed consent and replied to a standard questionnaire to determine the profile and habits (age, time of work, salary, type of diet most commonly practiced, type of housing, use of drugs and medications, etc.). The study was conducted with the approval of the Institutional Ethical Committee.

Chromosomal aberration (CA) assay. All chemical reagents were purchased from Sigma Chemicals (St. Louis, MO, USA), except for colcemid that was obtained from Gibco Laboratory (Grand Island, NY, USA). Upon arrival in the laboratory, blood samples were set up to establish cell cultures following standard procedures in the laboratory (Moorhead *et al.,* 1960). Briefly, 0.5 ml of whole blood was added to 4.5 ml RPMI 1640 medium, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% streptomycin–penicillin antibiotics, 0.2 ml reagent grade phytohemagglutinin, and incubated at 37°C. At 71st hr, cultures were treated with 0.1 μg/ml colcemid to block cells in mitosis.

Lymphocytes were harvested at 72 h by centrifuging cells to remove culture medium (800–1 000 rpm/7 min), and adding hypotonic solution (KCl 0.075 M) at 37°C for 20 min to induce cell swelling, following the treatment with fixative (3:1 ratio of methanol:acetic acid), which was then repeated. Cytological preparations were made by adding 2–3 drops of the concentrated cell suspension onto slides rinsed with ice-cold acetic acid (60%). Slides were carefully dried on a hot plate (56°C, 2 min). For CA analysis, 100 complete metaphase cells were evaluated per subject under a microscope (100x) to identify numerical and structural CA.

Micronucleus (MN) assay. The MN assay was performed using the cytochalasin B technique (Fenech & Morley 1985). Lymphocytes were cultured as described above. Cytochalasin B (6 μg/ml) was added at 44 h of incubation. After a total incubation time of 72 h at 37°C, the cells were harvested by centrifugation, rinsed, and submitted to mild hypotonic treatment followed by immediate fixation with methanol: acetic acid. Slides were prepared according to standard cytogenetic procedures and stained with 4% Giemsa. Slides were then coded and scored by light microscopy at 400x magnification. For each experiment, 1 000 binucleated lymphocytes with well-preserved cytoplasm were scored. MN was identified according to the criteria of Fenech *et al. (*2003).

Genotypic analysis for *XRCC1* 399 Arg/Gln polymorphism. The whole genomic DNA was collected as per kit protocol (Bangalore Genei-Frozen blood DNA extraction kit) for PCR analysis (Duell *et al.,* 2000). Cleavage with polymorphism was carried out using MspI for Arg399Gln (Lei *et al.,* 2002).

Statistical analysis. A parametric statistical test was carried out in the study. The statistical significance of the difference in the frequency of XRCC1 genotype between groups was calculated by X^2 test. The analysis was performed using SPSS (version 13), and ultimately confirmed using ANOVA within and between the groups of exposed subjects and controls.

Table 1. General characteristics of coke oven workers and control subjects

Characteristics	Number of subjects n = 54		Percentage (%)
	Exposed subjects	Controls	
Group I (<35 years)	12	12	44.4%
Group II s(≥35 years)	15	15	55.5%
Gender			
Male	27	27	100%
Smoking			
Yes	13	13	48.1%
No	14	14	51.8%
Work duration			
<12 years	11	NA	40.7%
≥12years	16	NA	59.2%

Refer to Table S1 for detailed raw data of Table 1. NA, Not applicable; Group I, (<35 years), Group II (≥35 years)

RESULTS

A total of 54 subjects, including 27 exposed and 27 controls, were recruited for our study. Their characteristics were listed in Table 1. The study includes group I 12 (44%) subjects and group II 15 (56%) for both exposed and controls, respectively, in which 13 (48%) were smokers and 14 (52%) were non-smok-

Table 2. Description of chromosomal aberrations and Micronucleus in coke oven workers in comparison with unexposed controls

Group	Age	Work duration	CA/100 cells		Total	MN/1 000 cells
			CTAs	CSAs		
Coke oven workers						
Group I (<35 years)	27.9±3.9	5.5±3.5	1.7±1.0*	0.25±0.4*	2±1.2*	1.9 ±2.7*
Group II (≥35 years)	44.4±5.8	18.2±4.5	3.4 ±1.0*	0.46±0.5	4.6±1.4*	6.6 ±2.2*
Controls						
Group I (<35 years)	27.5±4.4	NA	0.33± 0.65	0.16± 0.38	0.5±0.6	1.0±0.9
Group II (≥35 years)	43.5±5.2	NA	1.26±0.79*	0.6±0.50	1.8± 0.9	2.8 ±1.3*
Coke oven Workers						
Smokes	29.5±5.3	18.9±4.4	3.4±1.1	1.4±0.6	4.9±1.7	6.6±2.6
Non smokers	45.2±5.9	6.6±4.3	1.7±1.0	0.5±0.6	2.3 ±1.1	3.3±2.1
Controls						
Smokes	29.2±5.7	NA	1.30±0.8	0.5±0.5	1.7±1.0	3±1.3
Non smokers	44.1±5.6	NA	0.42±0.6	0.2±0.4	0.7±0.7	1.1±0.9

*values significant at $p < 0.05$ level by ANOVA; Refer to Table S1 for detailed raw data of Table 2; CTAs, chromatid type aberration; CSAs, chromosomal type aberrations; MN, Micronucleus; Group I (<35 years), Group II (≥35 years); NA, Not applicable. *Work duration <12 year / ≥ 12 year.

ers. The exposed subjects and controls were matched for gender (100% males) and age (±2 years relaxed). In our study, significant increase in CA and MN frequencies were observed in exposed subjects employed for a long time (group II — 18.2 ± 4.5) when compared to short duration workers (group I — 5.5 ± 3.5). Table 2 shows higher frequency of total CA in exposed subjects (4.6 ± 1.4) compared to controls (1.8 ± 0.9); similarly, the MN frequency was higher in exposed subjects (6.6 ± 2.2) compared to controls (2.8 ± 1.3). Statistically significant results were obtained for both CA and MN in exposed subjects, compared to controls (ANOVA). A higher degree of MN frequency was observed in smokers (coke oven workers) (6.6 ± 2.6) compared to non-smokers (3.3 ± 2.1). Also, the MN frequency in smokers was significant (3 ± 1.3) when compared to non-smoking (1.1 ± 0.9) exposed subjects. Table 3 shows allele frequencies of XRCC1 399Gln polymorphism of both exposed and control subjects, with insignificant results in exposed (0.320) and control subjects (0.381). XRCC1 is involved in the efficient repair of DNA single stand breaks. Regression analysis was carried out in XRCC1 399Gln frequency in coke oven workers and the results were tabulated.

DISCUSSION

PAHs have been identified as a major source of carcinogenic risk among coke oven workers (IARC, 1987). Exposure to high concentrations of PAHs in the environment and tobacco smokers have elevated levels of aromatic DNA adducts in blood cells (Eder, 1999). Chromosome defects are recognized as being the basis of a number of human genetic diseases. The frequency of CA in peripheral blood lymphocytes is a relevant biomarker for cancer risk in humans, reflecting either early biological effects of genotoxic carcinogens or individual cancer susceptibility (Heussner *et al.,* 1985). Our findings support these earlier studies, as they include higher values in the exposed subjects.

According to Bloomfield (Bloomfield *et al*., 1987), there is a positive correlation between CA frequency and increased cancer risk. Many investigators showed that the frequency of CAs in PBLs is a relevant biomarker for cancer risk in humans (Hagmar *et al.,* 1998). According to Agova *et al.* (2005), most commonly detected abnormalities were gaps and breaks, which are increased in the coke oven workers. The main relevance of lymphocyte chromosome damage is its indicative value for genotoxic exposure, which is considered to be related with the multistage process of carcinogenesis (Hartmann, 1983; Hagmar *et al.,* 1994; Smerhovsky *et al.,* 2001; Norppa *et al.,* 2006). Several studies have reported the relationship between aging and structural CA. The CAs in controls might be due to factors like age and lifestyle. Some studies have found a significant influence of age on CA frequency (Ramsey *et al.,* 1995; Tucker & Moore, 1996). The control subjects of group I and II showed minimum number of CA when compared to the exposed subjects. An age-related increase in the aneuploid cells of human lymphocytes has been reported in a number of studies (Nowinski *et al.,* 1990). Moreover, the results of the present study also points out the role of age in CA, which was supported by the study of Balachandar and coworkers (Balachandar *et al.,* 2008). Our study reveals that increase in age; both in exposed and control group shave significantly higher total CA.

MN assay in lymphocytes was suitable to detect the PAHs induced chromosome damage in coke oven workers exposed to high levels of PAHs (Burgaz *et al.,* 1995; Bonassi *et al.,* 2001; Leng *et al.,* 2004). According to Pa-

Table 3. XRCC1 genotypes and allele frequencies in coke oven workers and controls

XRCC1 399	Total number of samples	Arg/Gln	399Gln frequency	Regression analysis		
				Slope(b)	y-Intercept(a)	Regression equation(y)
Coke oven workers	27	58 (53.70%) p = 1.000 OR = 1 95% CI = 0.564–1.773	0.320	0.814	–0.094	–0.094 + 0.815
Controls	27	50 (46.29%)	0.381	0.774	–0.084	–0.084 + 0.775

CI, confidence interval; OR, odd ratio; XRCC 1, X-ray cross complementation gene 1; Gln, glycine; Arg, arginine; p, probability

Table 4. Pearson's correlation analysis of coke oven exposure to PAHs, age, and smoking habit with the levels of CA and MN

		Group 1 or 2	Smoking habits	Chromatid type alterations	Chromosomal type alterations	Micronucleus changes
Group 1 or 2	Pearson Correlation	1	.713**	.660**	.678**	.688**
	Sig. (2-tailed)		.000	.000	.000	.000
	N	27	27	27	27	27
Smoking habits	Pearson Correlation	.713**	1	.612**	.578**	.576**
	Sig. (2-tailed)	.000		.001	.002	.002
	N	27	27	27	27	27
Chromatid type alterations	Pearson Correlation	.660**	.612**	1	.598**	.931**
	Sig. (2-tailed)	.000	.001		.001	.000
	N	27	27	27	27	27
Chromosomal type alterations	Pearson Correlation	.678**	.578**	.598**	1	.698**
	Sig. (2-tailed)	.000	.002	.001		.000
	N	27	27	27	27	27
Micronucleus changes	Pearson Correlation	.688**	.576**	.931**	.698**	1
	Sig. (2-tailed)	.000	.002	.000	.000	
	N	27	27	27	27	27

**Significant at 0.01 level (2-tailed)

vanello *et al.* (2008), coke oven workers who were heavily exposed to PAHs (80% of workers exceeded the urinary 1-pyrenol biological exposure index value) presented significantly higher MN frequency in PBLs relative to control subjects. Our study supports the above findings by showing higher MN frequency in smokers of group II exposed subjects, which depicts a significant result. According to several investigations (Carrano & Natarajan 1982; Motykiewicz *et al.,* 1992; Bender *et al.,* 1992; Buchet *et al.,* 1995; Judson & Stephens, 2001; Bindhya *et al.,* 2010), XRCC1 399Arg/Gln polymorphism does not alter the affinity of XRCC1 for polynucleotide kinase/phosphatasepoly ADP-ribose polymerase among PAHs exposed workers. Our study highlights that there was no relationship between coke oven exposure and XRCC1 399 Arg/Gln polymorphism. Leng *et al.* (2005) reported a statistically non-significant additive interaction between the XRCC1 399Gln allele and PAHDNA adducts as XRCC1 genotypes. The result shows that CA, MN, and PCR-RFLP were found to be sensitive biomarkers in detecting the effect of exposure to PAHs.

Individuals with occupational exposure to toxic substances such as PAHs were given priority for investigation. The present study demonstrates that exposure to coke oven emissions constitutes an increased carcinogenic risk and confirmed that applied cytogenetic markers constitute a useful tool for determination of these risks. Thus our findings indicates that, the exposed smokers in coke oven industry and slow acetylators were more prone to genetic damage. It was concluded that coke oven workers at risk should be carefully monitored snd should take adequate protective measures to prevent long-term adverse effects. We found that, coke oven workers, occupationally exposed to PAHs, shows significantly increased levels of biomarkers, especially CA and MN, which were found to be more sensitive for biomonitoring the PAHs exposure at occupational settings.

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