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Assessment of DNA Damage by Comet Assay in Lymphocytes of Workers Occupationally Exposed to Petroleum fumes

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Abstract- The comet assay is a sensitive, reliable and rapid method for DNA double & single strand break, alkali-labile sites and delayed repair site detection in individual cells. In recent years, this method has been widely used for studies of DNA repair, genetic toxicology, environmental biomonitoring, and apoptosis. However, this technique serves as an important tool for detection of DNA damage in living organisms and is increasingly being used in genetic testing of industrial chemicals, agrochemicals & pharmaceuticals. This research paper helps to evaluate the health effects of exposure to organic pollutants by using comet assay technique by KOMET 3.1 software. This study used human biomonitoring to evaluate the genotoxic effects of exposure to organic pollutants. The experimental data suggest that the DNA damage parameters (OTM, % T-DNA and TL) were found higher value in exposed population when compared to the control healthy population. The percentage and distribution of cells in exposed population also increases with the increase in OTM values. This study demonstrates that, using sensitive techniques, it is possible to detect human health risks at an early stage when intervention is possible.
Key Words- Comet assay, DNA damage, Komet 3.1, Single cell gel electrophoresis, Genetic toxicology, Environmental biomonitoring, Olive Tail Moment, % Tail-DNA, tail length.

INTRODUCTION

In recent years, SCGE, referred to as the 'comet assay' after the shape of the images (comets) seen under the microscope, has been widely used to detect DNA strand breaks, alkali-labile sites, DNA crosslinking, and incomplete excision repair sites. It is a microgel electrophoresis technique which permits the measuring of DNA damage cell by cell. The alkaline version, introduced by Singh et al. [1], detects DNA breakage, alkali labile sites, open repair sites and crosslinks. For this technique, cells are mixed with molten agarose, which is spread onto a microscope slide. The cells are then lysed with high salt concentrations and detergents. The remaining nuclear DNA is then denaturated in an alkali buffer and electrophoresed in the same buffer. The DNA fragments migrate from what was the nucleus towards the positive electrode. After electrophoresis, the slides are stained with a fluorochrome for UV imaging of the DNA. An image-analysis system is used to measure several damage parameters. The most useful are tail length (TL), measured from the centre of the nucleus towards the end of the tail, the percentage of DNA in the tail (TD), and Olive Tail- Moment (OTM = TL x TD). This technique has suggested a positive role of the comet assay in the human monitoring of DNA damage from environmental and/or occupational exposure to carcinogenic and mutagenic agents, and has been shown to be a very sensitive method to detect genetic damage at the individual cell level and in human biomonitoring [2]. Owing to its sensitivity in detecting genetic damage at the individual cell level and its potential application to virtually all eukaryotic cell types, the assay has been adopted as a useful tool in short-term genotoxicity and human biomonitoring studies [3]. The widespread use of petroleum products by industry and in automobiles has resulted in the deterioration of air quality, leading to exposure of the general population. Individuals who are employed as petrol-pump workers (PPWs) are constantly exposed to automobile exhaust as well as petroleum fumes. Earlier studies demonstrate that PPWs suffer from headaches, sleep disturbance, memory loss, and general weakness [4-5]. PPWs also excrete high levels of phenol in their urine indicating exposure to benzene [5]. Trans muconic acid has also been used as an indicator of environmental benzene exposure [6]. These observations indicate that the continuous monitoring of personnel at petrol stations, as well as the surrounding air, is warranted due to the occurrence of known or suspected animal and human carcinogens in petroleum products (4, 7-9]. PPWs do not wear personal protective equipment and are chronically exposed to petroleum derivatives primarily through inhalation of the volatile fraction of petrol during vehicle fueling. As a result of inhaling petrol fumes, benzene can be absorbed in the lungs. Benzene levels in human breath [10] and blood [11] are elevated after refueling at self-service petrol stations. This suggests that PPWs, who are constantly fueling vehicles, are at an elevated risk to the adverse effects of benzene. Monitoring of occupational exposure to chemicals is important in evaluating risks and implementing strategies for improving occupational safety and health. The use of the Comet assay [12] to

measure DNA damage and genotoxicity in human population provides information on possible early biological effects of exposure and may be indicative of health risks. The data of healthy subjects from our earlier studies on the DNA damage in human lymphocytes (13-16] have now been considered as reference values for the Indian population [17]. Human biomonitoring studies conducted in developed countries, as well as in China and Thailand, have established associations between elevated levels of DNA damage and exposure to urban air pollution [18-20]. However, the genotoxic effect of benzene in petroleum in the Indian population has not been studied. Hence, it was considered prudent to assess biomarkers of exposure and effect in an Indian population exposed to petroleum fuels. In the present study, we have used the Comet assay on lymphocytes from Indian PPWs to evaluate the genotoxic effects of occupational exposure to petroleum derivatives. If this was found to be present, the level of DNA damage, as well as the level of DNA repair, was to be determined. The genotoxic potential of an environment where petroleum derivatives were present, was therefore investigated.

MATERIALS AND METHODS

Chemicals

The chemicals used in the study were of analytical grade (purity > 98%). Phosphate-buffered saline (PBS; Ca^{2+}, Mg^{2+} free), low melting point agarose (LMA), and RPMI-1640 were purchased from GIBCO-Invitrogen (Carslbad, CA). Normal melting point agarose (NMA) and Triton X-100 were obtained from Hi Media (Mumbai, India). Ethidium bromides, ethylenediaminetetracetic acid disodium salt, Tris buffer, Trypan blue, were obtained from Sigma (St. Louis, MO). All other chemicals were obtained locally.

Selection of Volunteers

Two hundred male volunteers (125 PPWs and 125 controls), from 20 automobile service stations in the city of Lucknow, India, met the criteria of the study. The inclusion criteria for the study subjects were as follows: the subjects were apparently healthy males between the age group of 18-58 years, with a minimum exposure of six months. The controls on the other hand were also males employed 200-500 m away from the petrol pumps and healthy. They too were between 18 and 65 years with no exposure to petroleum fumes and matched for lifestyle (e.g., smoking, tobacco-chewing, consumption of alcohol), and diet. All the subjects had no past history of disease, and were not taking any medication and comprised a mixed population, not representing any particular ethnicity. Informed consent was obtained from all the volunteers and the study was conducted in accordance with the Institutional Medical Ethics Committee of the Institute. The volunteers were interviewed to obtain factual information on their occupation and personal habits. They answered a detailed questionnaire about their dietary habits (vegetarian and nonvegetarian diet), lifestyle (e.g., smoking, tobacco-chewing, consumption of alcohol), and occupation. Blood was taken only from those individuals who were asymptomatic and were not on any medication. The sampling was done towards the end of the work week and not on the day after the worker was on leave.

Alkaline Comet Assay

Blood samples were collected by finger prick into heparinized tubes stored on ice. The tubes were protected from light. Lymphocytes were isolated from whole blood using Histopaque 1077 as described by Pandey et al. [16]. Viability was determined by the Trypan blue dye-exclusion technique before conducting the Comet assay [21]. Slides were prepared and the alkaline Comet assay conducted as described by Pandey et al. [16]. In general, the assay was conducted according to the method of Singh et al. [1] and the guidelines of Albertini et al. [22]. Briefly, the lymphocytes were mixed in LMA at 37 ^{0}C, and layered on to a precoated end-frosted slide. A cover slip was placed on the slide to ensure that the gel was evenly spread, and the slide was kept on ice to allow the gel to solidify. A third layer of low melting agarose was added and again allowed to solidify on ice. Duplicate slides were prepared for each sample. Finally, the cover slips were removed and the slides immersed in freshly prepared and chilled lysis solution (containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris (pH 10) with 1% Triton X-100 being added just before use. The slides were kept overnight at 4 ^{0}C. After lysis, the slides were placed in a horizontal gel electrophoresis tank (Life Technologies, USA) containing fresh and chilled electrophoresis solution (1 mM Na_2EDTA and 300 mM NaOH, pH > 13) to allow DNA unwinding (20 min), and electrophoresis was conducted for 30 min at 48 ^{0}C at 0.7 V/cm and 300 mA (power supply Electrablot III from Techno Source India Pvt. Ltd. Mumbai, India). All steps were performed under dimmed light. After electrophoresis, the excess alkali was neutralized with Tris buffer (0.4 M Tris, pH 7.5), and slides were stained with 75 µL ethidium bromide (20 µg/mL). Slides were stored in a dark humidified chamber until analysis. Slides were scored using an image analysis system (Kinetic Imaging, Liverpool, UK) attached to a fluorescence microscope (Leica, Germany) equipped with appropriate filters (N2.1). The microscope was connected to a computer through a charge coupled device (CCD) camera to transport images to software (Komet 3.1) for analysis. The

final magnification was 400X. One hundred cells per individual were randomly scored. The parameters used as metrics of DNA damage were Olive tail moment (OTM; arbitrary units), tail DNA (%), and tail length (μm).

Statistical Analysis

The mean values of the Olive tail moment among the various groups were compared using students't' test. Prior to analysis, homogeneity of variance between treatment and normality assumption of data was tested. Tail DNA (%) and tail lengths were also analysed separately for significance. Frequency distributions of cells in each class interval were compared using chi square (χ^2) tests.

RESULTS

The general characteristics of the volunteers (125 PPWs and 125 controls) are summarized in Table 1. The mean age of the controls was significantly higher than that of the PPWs ($P < 0.05$; Table 1). The lymphocytes from control and PPW subjects were more than 90% viable at the time of the assay. Lymphocytes from PPWs exhibited a significantly ($P < 0.001$) greater OTM (4.14 ± 1.25) compared with the controls (3.29 ± 0.92). A similar significant increase in tail DNA (%; $P < 0.001$) and tail length (μm; $P < 0.01$) was observed for the PPW samples (Table 2). The DNA damage is measured by the parameters Olive tail moment (OTM), % tail DNA and tail length illustrated in figure 1. The exposed and control groups were stratified by age into three groups of <30, 31–40, and >40 years (Table 2). Amongst the controls or the PPWs, there was no significant effect of age on the Comet assay responses. Within each of the age categories, however, the PPWs had significantly more DNA damage than the controls. The Comet assay responses from PPWs also were stratified, according to the duration of exposure, into groups with <5, 6–10, 11–15, and >15 years of employment as PPWs. Although the values of the Comet assay parameters tend to become smaller with increased exposure, none of the differences were significant (Table 2).

DISCUSSION

The Comet assay has been widely used for the sensitive assessment of DNA damage in occupationally and environmentally exposed populations [23-24]. This study revealed a significant increase in DNA damage in lymphocytes of PPWs exposed to petroleum fumes in comparison to controls as evident from the Comet parameters OTM, tail DNA (%), and tail length. Previous studies measuring the genotoxicity of petroleum fuels in humans have been conducted in developed countries [23, 25-28]; however, this is the first such study in India. The present study was conducted to evaluate the health effects of exposure to petroleum fuels and to assess if petroleum fuels have any genotoxic effects. Our data suggests that the damage to DNA in lymphocytes can be related to the exposure to petroleum fuels as shown by the increase in the olive tail moment (OTM), percent tail DNA (% T-DNA) and tail length (TL) values in exposed individuals. The percentage distribution of the cells according to the OTM, indicates that there is a significant increase in the percentage of cells showing higher damage in the volunteers exposed to petroleum fuels. In conclusion, our study shows that the people exposed to petroleum products exhibit genotoxicity.

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Table 1- Characteristic Profiles of the Control and Petrol-Pump Workers (PPWs) Evaluated for DNA Damage in the Comet Assay

	Controls	PPWs
Total number of subjects	125	125
Age (years)	30.9 ± 10.7* (18–65)	27.4 ± 8.5 (18–58)
Body mass index (BMI)	21.9 ± 4.6* (14.9–34.4)	20.7 ± 3.4 (15.9–32.8)
Duration of exposure (years)	Nil	7.3 ± 7.1 (0.5–35)
No. of smokers	30	29
No. of tobacco chewers	45	58
No. of alcohol drinkers	26	21

*P < 0.05 significant when compared with PPW.
When not specified as number of subjects, values are mean ± SD (range)

Table 2- Effect of Age and Years of Exposure on Comet Assay Parameters for Lymphocytes from Controls and Petrol-Pump Workers (PPWs)

Parameters	Control (n =125)			PPWs (n = 125)		
	OTM (arb. units)	Tail DNA (%)	Tail length (µm)	OTM (arb. units)	Tail DNA (%)	Tail length (µm)
Age Unadjusted	3.3 ± 0.9	10.1 ±1.8	53.2 ± 11.7	4.2 ± 1.3**	12.2 ±2.6**	58.7 ±12.2*
Age Stratified by years						
< 30 years of age	3.29 ± 0.95	10.2 ± 1.9	53.24 ± 12.1	4.1 ± 1.2**	12.2 ± 2.6**	57.9 ± 11.9*
31-40 years of age	3.25 ± 0.74	9.98 ±1.67	54.27 ± 9.85	4.3 ± 1.3**	12.1 ± 2.0**	60.9 ± 11.7*
> 40 years of age	3.36 ± 1.07	9.88 ±1.84	51.71 ± 13.0	4.4 ± 1.6	11.9 ± 3.16	61. 3 ± 15.2
Exposure in PPW						
< 5 years				4.22 ± 1.26	12.19 ± 2.49	58.5 ± 12.9
6–10 years				4.21 ± 1.20	12.56 ± 3.06	58.7 ± 7.3
11–15 years				3.99 ± 1.30	11.75 ± 2.42	58.8 ± 14.3
>15 years				3.98 ± 1.36	11.74 ± 2.44	59.8 ± 15.1

All the values are mean ± S.D., *P < 0.05, **P < 0.001 when compared with controls.

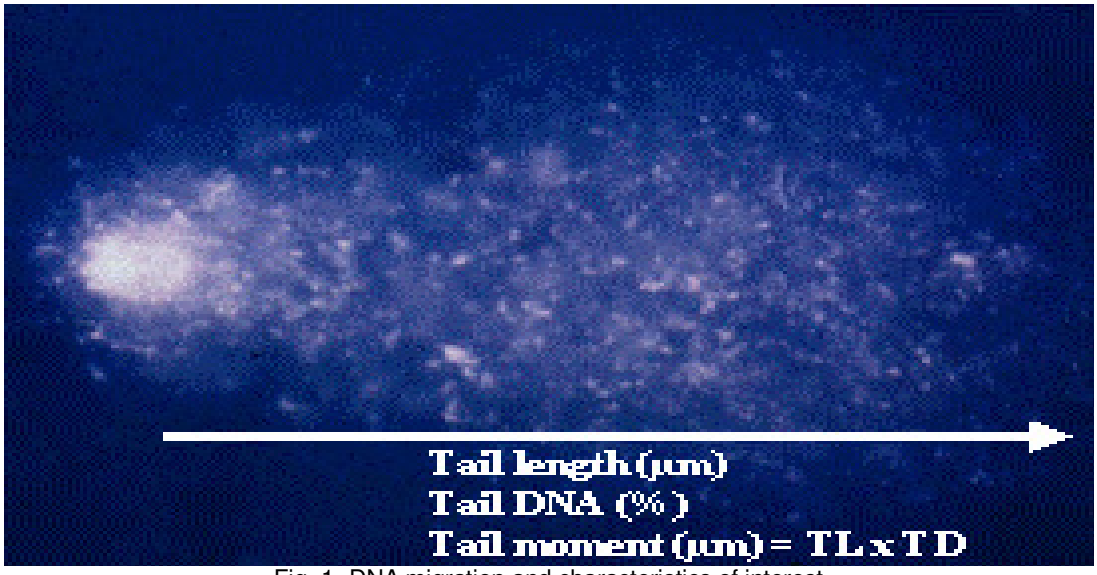

Fig. 1- DNA migration and characteristics of interest

