

Genotoxic effects of occupational exposure to benzene in gasoline station workers

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Abstract: Benzene, a hazardous component of gasoline, is a genotoxic class I human carcinogen. This study evaluated the genotoxic effects of occupational exposure to benzene in gasoline stations. Genotoxicity of exposure to benzene was assessed in peripheral blood leucocytes of 62 gasoline station workers and compared with an equal numbers of matched controls using total genomic DNA fragmentation, micronucleus test and cell viability test. An ambient air samples were collected and analyzed for Monitoring of benzene, toluene, ethyl benzene and xylene (BTEX) in work environment and control areas. DNA fragmentation, micronucleus and dead cells percent were significantly higher in exposed workers than controls. Level of benzene, Toluene, Ethyl benzene and xylene in the work environment were higher than the control areas and the permissible limits. Gasoline station workers occupationally exposed to benzene are susceptible to genotoxic effects indicated by increased DNA fragmentation, higher frequency of micronucleus and decreased leukocytes viability.

Key words: Benzene, Genotoxic effect, DNA damage, Micronucleus, Cell viability

Introduction

Occupational exposure to chemical hazards and toxic substances can cause a variety of health hazards ranged from irritation to carcinogenicity. Gasoline station workers who are a part of fueling and refueling of vehicles are at higher risk of adverse health effects¹. Gasoline station workers are exposed to volatile organic compounds such as benzene, toluene, ethyl benzene and xylene (BTEX) from fuel vapors during dispensing fuel, in addition to emissions from vehicle exhausts. They are directly exposed to BTEX compounds through inhalation and dermal contacts². However, the main route of exposure is the respiratory

system. Benzene is well known genotoxic group 1 human carcinogen while ethyl benzene is classified as a possible human carcinogen group 2B³. Also, hemato-lymphoid toxicity, including pancytopenia, aplastic anemia, myeloid and myelodysplastic leukemia could be related to benzene exposure^{4,5}. Ethyl benzene and xylene have respiratory and neurological effects⁶.

DNA fragmentation is considered as an indicator of possible genotoxicity. Micronuclei (MN) are chromosomal materials that originated from acentric fragments of DNA or complete chromosomes that failed to attach the mitotic spindles. Therefore, the occurrence of MN constitutes a relatively simple and direct assay for screening risks of genetic damage in individuals occupationally exposed to mutagenic agents. MN assay is considered to be a reliable method that responds to any genotoxins⁷. Classical cytological tests illustrated that benzene exposure induces

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DNA damage⁸). Although no hematological disturbance was found after indoor exposure to petroleum derivatives, genotoxicity is evident⁹. The increased micronucleus frequency and chromosomal aberrations in the occupationally benzene and its metabolites exposed people are evident⁸ in addition to sister-chromatid exchanges in cultured human lymphocytes¹⁰.

Records of occupational genotoxicity due to the exposure to petroleum products in gasoline stations from Egypt are infrequent. Therefore, the present study was designed to investigate the genotoxicity due to occupational exposure to benzene in gasoline station workers using DNA fragmentation, micronucleus assay and cell viability in addition to the estimation of BTEX levels in the work environment.

Participants and Methods

Study setting

This was a comparative cross-sectional study conducted from the beginning of May to the end of December, 2016 at all licensed gasoline stations (16 stations) in Shebin El-Kom city, Menoufia Governorate, Egypt. Shebin El Kom is the capital of Menoufia governorate and had the largest numbers of gasoline stations in the governorate.

Study population

Out of ninety-eight gasoline station male workers in all studied stations, sixty-two exposed workers were illegible to participate in this study after application of exclusion criteria with a response rate of 89.7%. All the workers in the present study were more than 18 yr old, worked in gasoline stations for more than one yr and elaborate in accomplishment responsibilities like filling and infilling fuel from tankers to filling pumps and pumping fuel into vehicles. No one of the workers used any face masks or hand gloves and worked round oil and grease.

An equal number of controls were recruited from the workers' relatives with no history of benzene occupational exposure, or any known genotoxic agent. They were matched with exposed workers regarding age, gender and socioeconomic standard.

It was also confirmed that the exposed and the control subjects had not been taking any medical treatments which could cause the DNA damage nor been exposed for twelve months before sample collection to any kind of radiation.

The Menoufia Faculty of Medicine Committee for Medical Research Ethics revised and officially approved the study before its beginning. Informed formal consent was

obtained from each applicant prior to the beginning of the study. All subjects involved in the study received thorough information regarding the aims of the research study. The approval from the governmental authorities was also done.

Methods

All participants were subjected to:

I. Interview questionnaire

An interview questionnaire was designed and conducted by the authors. The questionnaire was developed by the researchers after review of related literature and was tested for content validity by a panel of experts in the field. Unclear and ambiguous wordings were modified based on the responses of these experts. It included demographic data (age, residence) as well as medical history (exposure to X-rays, vaccinations, medication), special habits (smoking, alcohol), and occupational histories (working h/d, yr of exposure, use of protective measures) A pilot study was carried out on ten subjects that were excluded from the study sample to test feasibility and applicability of the tools and modifications were done accordingly.

II. Monitoring of BTEX in ambient air by gas chromatography

The exposure to BTEX in ambient air was monitored during work shifts of eight h. Ambient air samples was collected at 1.5 meters above ground about 2–3 meters from the fuel pump by active sampling with a flow rate 100 ml/min using SKC battery operated air sampling pump model PCXR4. Activated charcoal cartridges were used to collect samples. At each station, two to three samples were collected at several time intervals (total 40 samples).

Two air samples were collected during day and night times (24 h) at fixed sites in a congested and non-congested street in Shebein El Kom city (as an urban control area). A third sample was collected from a rural area in Menoufia governorate. The mean value of the three samples was taken as the control areas.

After sampling, the contents of each cartridge were placed in a separate vial which was sealed and placed in a cooled path for 20 min and then left for 1 h at 10°C. The samples were extracted with carbon-disulphide (CS₂), and the sample solution was then analyzed by Gas Chromatography. The analysis procedure was modified from the NIOSH 1,501 method¹¹. VF-5MS column (30 m length, 0.25 mm internal diameter, 0.25 Pm film thickness) was used for chemical analysis. The column oven was pro-

grammed initially at 40°C for 3 min followed by heating rate of 15°C min⁻¹ ramp to a final temperature of 200°C. The final temperature was hold for 6 min. The average concentrations of BTEX compounds were calculated from all samples.

The BTEX calibration was done using a standard solution (Supelco EPA TO-1 Mix 1A). Solutions with concentrations ranging from 0.1 to 4.0 ng PL⁻¹ were used to build the calibration curve. The correlation coefficients were always above 0.99. The quantification limit calculated for each BTEX was 20 pg PL⁻¹, in relation to a concentration of 1.0 Pg m⁻³ in the atmosphere. All these steps were done under the supervision of qualified professor from air pollution department, at the national research center, Cairo, Egypt.

III. Measures of genotoxicity

About 5 ml of venous blood sample was collected from each participant using sterilized syringes and then transferred to K-EDTA containing tubes. After labeling and processing the samples, they were transported to the laboratory within three to four h.

Peripheral blood leukocytes were isolated by incubation with four volume folds of erythrocyte lysing buffer (0.015 M NH₄Cl, 1 mM NaHCO₃, 0.1 mM EDTA). Then, they were centrifuged for 5 min at 1,000 rpm using cooling centrifuge (Sigma 3K 30, Germany). These steps were repeated until a white pellet appeared¹². The samples were investigated for the following:

a. Total genomic DNA extraction and agarose gel electrophoresis

DNA extraction (DNA fragmentation assay) was done by "salting out extraction method"¹³ with some modifications¹⁴. The method of extraction is summarized as follows: cells were lysed with 0.5 ml lysing buffer (10 mM Tris base, 10 mM NaCl, 10 mM Na₂ EDTA, 0.5% SDS, pH 8) overnight at 37°C then; 4 M NaCl was added to the specimens. Centrifuge the mixture at 10,000 rpm for 10 min. The supernatant was transferred to a new tube then DNA was precipitated by 1 ml isopropanol (cold) by centrifugation for 5 min at 12,000 rpm. Wash the pellets with 70% ethanol. Suspend the pellets in TE buffer (10 mM Tris, 1 mM EDTA, pH 8). Incubate for 30–60 min with loading mix (0.1% RNase+loading buffer), and then loaded into the agarose gel. Gels were prepared using 1.8% normal melting agarose in Tris borate EDTA buffer (89 Mm Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) for 1 h at 50 volts. The released DNA fragmentation appeared

against DNA marker (100–3,000 bp). The intensity of DNA fragmentation was measured by (ImageJ software) as a mean optical density values.

b. Micronucleus test assay by acridine orange fluorescent staining

Micronucleus Test (MN Test) is an assay used for screening genotoxicity by evaluating the evidence of micronuclei and other nuclear abnormalities in interphase cells cytoplasm. Peripheral blood mononuclear cell (PBMC), any blood cells with round nucleus, such as lymphocyte, monocyte or macrophage only was considered for MN test. One μ l of (1:1) acridine orange (50 μ g/ml)/ethidium bromide (5 μ g/ml) was added to five μ l of cell suspension on a clean glass slide. An experienced observer, blind to the sample groups, immediately observed the slides under 100 \times optic magnification using (Olympus BX 41, Japan) microscope equipped with 450–490 nm fluorescence filters¹⁵. Using scoring criteria¹⁶, the number of MN occurrences per 500 green viable observed cells per slide were identified and counted then representative digital microphotographs were captured.

c. Viability detection by acridine orange/ethidium bromide dual fluorescent staining

Nine μ l of peripheral leukocytes suspension were mixed with one μ l of dye - mixture (1:1) of acridine orange (50 μ g/ml)/ethidium bromide (5 μ g/ml) on a clean glass slide, then cells were immediately viewed under fluorescent microscope (Olympus BX 41, Japan) at 40 \times magnification. About 500 cells were observed and counted in each sample. Two types of cells were identified, based on emitted fluorescence, viable cells that had uniform bright green color with intact structure and late apoptotic or dead cells had orange to red color with chromatin condensation or fragmentation¹², and the representative photos were digitally photographed.

Statistical analysis

Data was analyzed with IBM SPSS Statistics software (version 20). All study variables were tested for normality by the Shapiro–Wilk test. Student's *t*-test and Mann–Whitney *U*-test was used to compare between study groups. The results were stated as mean \pm standard deviation (SD). Correlation tests were performed per Pearson's correlation coefficient and Spearman's rank according to each variable. Values of ($p \leq 0.05$) were considered significant.

Table 1. Mean ± SD concentrations of BTEX compounds (PPM) in gasoline stations and

BTEX	Gasoline stations Mean ± SD	Control areas Mean ± SD	ACGIH, 2010 Mean ± SD
Benzene	3.69 ± 1.88	0.05 ± 0.01	0.5
Toluene	120.59 ± 1.17	0.11 ± 0.02	100
Ethyl Benzene	133.70 ± 7.20	0.02 ± 0	100
Xylene	114.35 ± 6.86	0.04 ± 0.01	100

PPM: Part per million

Table 2. Characteristics of gasoline station workers and controls

Characteristics	Gasoline station workers (n=62)		Controls (n=62)		Test of significant	p
Age (yr):					t=1.57	0.119
Mean ± SD	34.47 ± 9.61		36.87 ± 7.27			
Residence:	No	%	No	%	χ ² =2.78	0.096
• Rural	28	45.2	19	30.6		
• Urban	34	54.8	43	69.4		
Smoking:					χ ² =2.21	0.138
• Smokers	44	71.0	51	82.3		
• Non-smokers	18	29.0	11	17.7		
Duration of smoking:					χ ² =1.67	0.319
Mean ± SD	15.15 ± 11.01		16.82 ± 7.17			

NB: There is no history of alcohol consumption in all the study participants.

Table 3. Comparison between DNA fragmentation, frequency of micronucleus and viability in gasoline station workers and controls

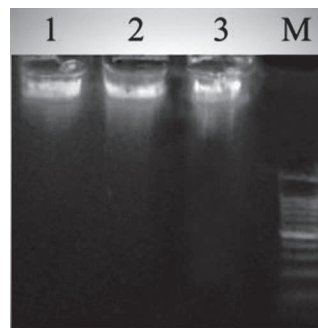
Items	Gasoline station workers (n=62) Mean ± SD	Controls (n=62) Mean ± SD	Mann-Whitney test	p
DNA fragmentation (OD)	23.39 ± 4.45	3.75 ± 0.87	8.57*	0.000
Micronucleus %	2.18 ± 1.33	0.45 ± 0.06	2.54	0.001
Dead cells %	15.25 ± 13.66	4.50 ± 0.58	1.54	0.020

OD: optical density OD: optical density and *t-test was used

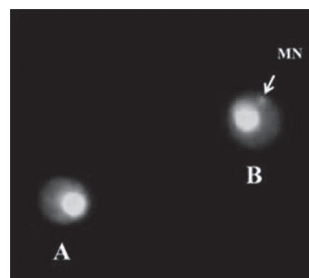
Results

Benzene, toluene, ethyl benzene and xylene (BTEX) exposure levels in gasoline stations were monitored by air sampling. Gasoline station workers were exposed to higher BTEX concentrations, since all values were above the ACGIH (American Conference of Governmental Industrial Hygienists) limits as shown in Table 1.

The gasoline station workers and controls were matched regarding their age and residence. More than half of gasoline station workers (71.0%) and controls (82.3%) were smokers, with no statistically significant difference between both groups about prevalence or duration of smoking, Table 2. There is no history of alcohol consump-



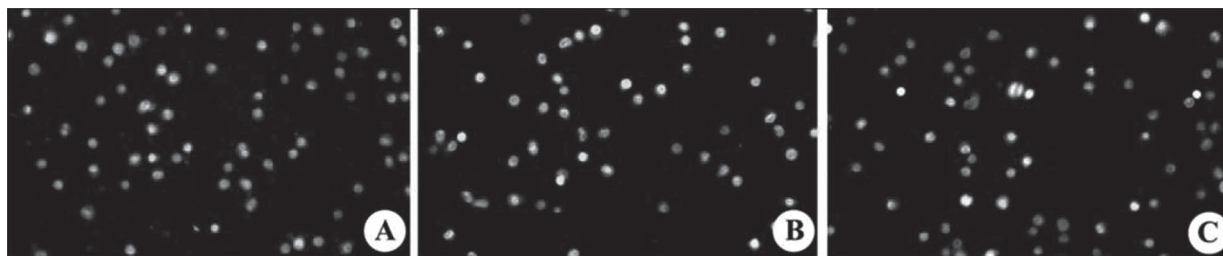
Photograph 1. Digital photography of total genomic DNA in peripheral blood leucocytes of gasoline station workers and controls. Where, 1: Control group, 2: Non-smokers gasoline station workers, 3: Smokers gasoline station workers and M: 100–3,000 bp marker.



Photograph 2. Micronucleated mononuclear Peripheral blood leucocytes (PBL) after ethidium bromide/acridine orange dual fluorescent staining method. Where A: normal cell and B: micronucleated mononuclear (PBL) (1,000×).

tion in all participants of this study. Specifically, mean ± SD of work duration among gasoline station workers was 8.63 ± 9.22 yr and the mean ± SD for working h/d was 9.22 ± 1.59. Concerning the use of protective measures, all the workers in gasoline stations were not using any of the protective measures (masks, goggles, or gloves) as it is not available in workplace.

On comparing the genotoxic markers in both exposed workers and controls as illustrated in Table 3 and photograph 1, gasoline station workers had significantly higher mean values of optical density of DNA fragmentation than controls (23.39 ± 4.45 and 3.75 ± 0.87; respectively). Concerning the frequency of micronucleus (Photograph 2), gasoline station workers had significantly higher mean percentage of micronucleus than controls (2.18 ± 1.33 and 0.45 ± 0.06; respectively). Regarding the viability of leukocytes (Photograph 3), gasoline station workers had significantly higher percentage of dead cells than controls (15.25 ± 13.66 and 4.50 ± 0.58; respectively). Moreover, smoker gasoline station workers (Photograph 3-C), had



Photograph 3. Photomicrography of viability of peripheral leukocytes stained ethidium bromide/acridine orange dual fluorescent staining method in controls (A), nonsmokers (B) and smokers (C) gasoline station workers (400×). The bright green color represents the viable leukocyte cells, while yellow to orange color represent the late apoptotic and dead cells.

Table 4. Comparison between DNA fragmentation, frequency of micronucleus and viability in smokers and non-smokers gasoline station workers

Items	Gasoline station workers		Mann-Whitney test	<i>p</i>
	Smokers (n=44)	Non-smokers (n=18)		
	Mean ± SD	Mean ± SD		
DNA fragmentation (OD)	23.10 ± 4.17	24.27 ± 6.17	0.38*	0.714
Micronucleus %	2.04 ± 1.33	2.57 ± 1.52	0.572	0.631
Dead cells %	18.11 ± 14.79	13.67 ± 12.08	1.61	0.110

**t*-test was used

Table 5. Correlations between parameters of DNA damage and age, years of smoking, duration of work and working hours in gasoline station workers*

	DNA fragmentation (OD)		Micronucleus %		Dead cells %	
	<i>r</i>	(<i>p</i>)	<i>r</i>	(<i>p</i>)	<i>r</i>	(<i>p</i>)
Ages (yr)	-0.249	0.435	0.245	0.443	0.132	0.683
Years of smoking*	-0.038	0.922	0.014	0.971	-0.229	0.554
Duration of work (yr)	-0.100	0.757	-0.120	0.711	0.052	0.872
Working h/d	0.469	0.124	-0.513	0.088	-0.507	0.079
Benzene (PPM)	0.357	0.001	0.317	0.003	0.212	0.032
Toluene (PPM)	0.155	0.165	0.123	0.261	0.142	0.159
Ethyle Benzene (PPM)	0.175	0.096	0.119	0.257	0.112	0.298
Xylene (PPM)	0.124	0.094	0.064	0.386	0.023	0.758

*In smokers (n=44)

increased percentage of late apoptotic and dead cells compared to non-smokers (Photograph 3-B) but not reached the significant levels.

On investigating the studied genotoxic markers in smokers and non-smokers exposed workers, no significant differences were found between the two subgroups regarding optical density of DNA fragmentation, frequency of micronucleus and viability of leucocytes as shown in Table 4. In addition, no significant correlations were found between the studied genotoxic markers and gasoline station workers' age, smoking years, duration of work or working h/d as shown in Table 5.

Discussion

Gasoline station workers are exposed to a combination of hydrocarbons in gasoline vapors during fuel supply that may harm their health. Monitoring of work environment and gasoline stations employee is anticipated to the known health hazards of petroleum products, especially benzene¹⁷.

All gasoline station workers in this study were males as our traditions in Arabic societies refused to engage females in these occupations where hard working, exposure to risks and night shift work.

In the current study, due to long exposure interval, the average levels of benzene (3.69 ± 1.88 ppm) was extremely higher in gasoline stations' environment as compared to the recommended exposure limits in Egypt (0.5 ppm over 8 h' exposure)¹⁸⁾ and in the USA, as the Occupational Safety and Health Administration (OSHA) permissible exposure level of benzene was 1 ppm. In addition, the National Institute of Occupational Safety and Health (NIOSH) recommended exposure level as 0.1 ppm¹⁹⁾. Also, the American Conference of Governmental Industrial Hygiene (ACGIH) Threshold Limit Value (TLV[®]) for benzene was 0.5 ppm²⁰⁾.

Moreover, the mean levels of Ethyl benzene, toluene and xylene in this study were extremely higher than TLVs (100 ppm for each)²⁰⁾. These results could reveal the poor safety procedures applied in the studied stations especially with the increasing number of the transporting vehicles. Significant higher mean levels of benzene, toluene, ethylbenzene, and xylenes were also reported in fuel filling stations from Thailand (11.28 ± 5.03 , 56.13 ± 73.96 , 7.17 ± 9.20 , and 10.59 ± 6.32 $\mu\text{g}/\text{m}^3$; respectively)²¹⁾, Brazil (Mean values 144.5, 157.0, 35.8 and 46.7 $\mu\text{g}/\text{m}^3$; respectively)²²⁾ and northern India (benzene, toluene and xylene were 7.94 ± 1.45 , 4.29 ± 0.69 , 5.10 ± 1.08 ppm; respectively)¹⁷⁾.

When vehicles are re-fueled, diesel vapors and diesel emitted exhausts from the vehicles may play a role in increasing the benzene concentrations inside the stations and are considered as the main sources of benzene in the atmosphere nearby²³⁾. Similarly, in Greece, the attendants of filling stations are still experiencing the exposure to high benzene concentrations ($15\text{--}52$ $\mu\text{g}/\text{m}^3$). Moreover, they found that benzene levels were directly proportional to the amount of dispensed fuel²⁴⁾.

In this study, DNA fragmentation and micronucleus frequencies, as genotoxic indicators, were significantly higher among gasoline station workers than controls. These results agreed with a study from North India¹⁷⁾ and in Brazil although benzene levels were lower than American Conference of Governmental Industrial Hygienist (ACGIH)²⁵⁾.

The genetic damage induced by occupational exposure to fuel vapors may be principally due to benzene with a considerable effect of co-exposure to toluene and xylene²⁶⁾. In addition, benzene occupational exposure may cause DNA damages⁸⁾ which could be the cause of micronuclei appearance¹⁷⁾. Moreover, once micronuclei are formed, they are not repairable and stand as a proof of genetic alteration that has occurred in dividing cells²⁷⁾.

Benzene toxic mechanisms are still elusive²⁸⁾. The results of benzene metabolism such as reactive oxygen

species (superoxide anion, hydrogen peroxide, hydroxyl radical) may be the main cause of biomolecules damage and may lead to DNA damage, micronuclei, and chromosomal abnormalities^{29,30)}. Moreover, DNA damage induced by benzene exposure could be due to (1,2,4-benzenetriol) as this is the ultimate metabolite of benzene which possess genotoxic potential on human lymphocytes¹⁰⁾.

The present findings showed that the percentage of dead cells was higher in gasoline station workers compared to controls. These results were in agreement with an Indian study²⁸⁾ conducted on 428 gasoline filling Indian workers occupationally exposed to benzene. The study revealed a significant reduction in lymphocyte number in benzene exposed workers compared to controls and the reduction was negatively correlated with years of exposure. Also, a negative correlation was found between decreased count of viable lymphocytes and increased concentrations of benzene (*in vivo* study). Moreover, it was found that, benzene metabolites (benzoquinone and hydroquinone) in concentrations above 100 μM , dose-dependently reduced cell survival³¹⁾. This finding might be due to the effect of benzene on programmed cell death induction (apoptosis) through generation of reactive oxygen species and oxidative stress or cell cycle alterations³²⁾.

The integrity of the genome is fundamental to the propagation of life, illustrated by the complexity of the DNA replication and repair mechanisms. As DNA damage is further induced by genotoxic stress. Failure to repair damaged DNA gives rise to mutations and chromosomal abnormalities. An important biological strategy to guard against damaged and mutated cells is the activation of programmed cell death³³⁾.

In the present study, although DNA fragmentation, micronucleus percentage and dead cells percentage of peripheral blood leukocytes were significantly higher in gasoline station workers than controls but these parameters were not significantly correlated with working years or working h/d. These results could be due to small sample size of workers in our study.

On the other hand, an increase in total comet score with an increase in exposure duration was described in a study recruited in Pakistan¹⁾. Also, a close relationship between workers' genotoxicity and duration of occupation was found in Canadian petroleum stations³⁴⁾. Moreover, subjects exposed for (5–10) yr or more have the highest ratio of DNA damage when compared to those exposed for (1–3) yr.

Age and smoking didn't affect the results for DNA damage found in this study, as no significant difference was

found between smokers and nonsmokers exposed workers regarding the studied genotoxic parameters also, age wasn't correlated with the studied genotoxic parameters. Also, Benites *et al.*³⁵⁾, reported that smoking and alcohol habits are not related to the levels of micronuclei and binucleated cells in gas station attendants.

On the contrary, Keretsetse *et al.*³⁶⁾, reported that age and smoking had a significant impact on the level of DNA damage in African petrol attendants but, DNA repair capacity was delayed in smokers of both exposed and unexposed group. Moreover, Kopjar *et al.*³⁷⁾, revealed that age and smoking significantly increased the values of MN evaluated by the cytokinesis-block micronucleus (CBMN) assay. The cut-off value, which resembles 95th percentile of the distribution of 200 individual values, was 12.5 MN. Other factors as diet, especially vitamin deficiencies and food supplementations (as) could affect MN levels and genetic damage³⁸⁾, These findings might explain the prevalence of MN in non-smokers.

The small sample size might limit the generalization of the results to all gasoline station workers, but it was unlikely to result in a significant impact on our findings concerning the association between benzene exposure and genotoxic effects in gasoline station workers. Also, the present study assesses only environmental exposure of benzene and lack of biological assessments as they weren't available. However, a previous study found well correlation between personal benzene exposure and urinary benzene metabolites over a broad range of exposure (0.06–122 ppm)³⁹⁾. The results of this study might be an important baseline data on genotoxic effects of BTEX exposure in gasoline station workers. These results should be verified in future large-scale studies.

Conclusion and recommendations

There were positive correlations between benzene exposure and DNA fragmentations, micronucleus formation and increased dead cell percentage of peripheral blood leucocytes in gasoline station workers. Age, smoking and duration of exposure had no significant influence on these genotoxic parameters. Wide scale researches, periodic monitoring of occupational exposure to chemicals as well as implementation of safety measures at gasoline stations including maintenance, prevention of chemical leak, matching the exposure limits with national and international permissible limits and applications of personal protective equipment as masks were recommended.

Conflict of interest

The authors declare that they have no conflict of interest

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