ORIGINAL ARTICLE

Cytogenetic bio-monitoring in fuel station attendants of Gujrat, Pakistan through buccal micronucleus cytome assay

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Abstract

Objective: To analyse buccal epithelial cells for scoring the frequency of cytogenetic toxicity in petrol pump workers.

Methods: The case-control study was conducted at the Lahore College for Women University, Lahore, Pakistan, from September 2014 to February 2015, and comprised petrol pump workers. Buccal micronucleus cytome assay was carried out by the collection of buccal cells after the written and oral consent of petrol pump workers. Frequencies of genotoxic (micronucleated and binucleated) and cytotoxic (karryorhetic and karyolitic) cells were scored and compared with the control group. The control group samples were collected randomly by keeping in view that these workers had not worked in petrol pumps ever. SPSS 16 was used for data analysis.

Results: Of the 200 participants, there were 100(50%) workers and 100(50%) controls. Statistically significant results were observed on the comparison of frequencies of different cell anomalies in subjects compared to controls on the basis of job duration, smoking habits and age ($p \leq 0.05$ each).

Conclusion: Pump workers showed higher frequencies of cytogenetic toxicity compared to controls.

Keywords: Buccal cell assay, Fuel station attendants, Gasoline, Genotoxicity, Micronuclei, Cytotoxicity. (JPMA 67: 1039; 2017)

Introduction

Fuel station attendants are exposed to several toxic compounds present in automotive fuels which are responsible for various numbers of toxicological manifestations in human beings.[1] Gasoline is the mixture of volatile polyaromatic hydrocarbons (PAHs). The aromatic portion of hydrocarbons is very complex and it contains some carcinogenic compounds, such as benzene, anthracene and benzo[a]pyrene (BaP). BaP is mainly of anthropogenic derivation and have no significant natural sources. It is persistent and not only present in the composition of both leaded and unleaded gasoline, but it is also released from vehicular exhaust. Among the different types of exposures to a genotoxic compound, occupational exposure is very important, as the individual is continuously and directly in contact with the compound.[2] Gasoline station workers are exposed to BaP through inhalation or by dermal contact if special safety actions are missing, i.e. personal protective equipment (PPE) is not used during fuelling and handling processes. The International Agency for Research on Cancer[3] has classified BaP as a cancer-causing compound in humans and other animals.

Genotoxicity biomarkers are used for the prediction, quantification and detection of various health risks resulting as consequences of exposure to chemicals in the environment.[4] Buccal cells symbolise a preferably first target site for early genotoxic events that are induced by carcinogenic agents entering the body. The buccal micronuclei cytome assay (BMCyt) is an excellent candidate to serve as a biomarker. To assess the cytogenetic damage, it is important to consider both genotoxic and cytotoxic parameters. Micronuclei (MN) are formed by abnormal chromosomal fragments, which appear on exposure to different types of deoxyribonucleic acid (DNA) damaging agents. Therefore, they are extensively acknowledged as an endpoint for the estimation of DNA damage.[5] Binucleated (BN) cell is most commonly present in cancer cells and may appear as a result of different type of causes; the increased rate of binucleation is usually not preferably a definite indicative parameter. Karryorhetic (KR) and karyolitic (KL) cells showed the cytotoxic state of the cell and may appear as a result of apoptotic or necrotic form of cell death due to increasing age and exposure to mutagenic or carcinogenic xenobiotics. This is a short-term assay for monitoring cytogenetic damage in humans and has been used to demonstrate effects of environmental exposure as a result of various occupational exposures, diseases, dietary deficiencies and lifestyle factors.[6] The current study was planned to carry out the questionnaire survey of the study population for their working hours per day,

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working span in pumps, socio-economic status, medical check-ups and usage of personal protective equipment, and to collect and analyse buccal epithelial cells for scoring the frequency of cytogenetic toxicity in petrol pump workers and comparing them with controls.

Subjects and Methods

The study was conducted at the Lahore College for Women University (LCWU), Lahore, Pakistan, from September 2014 to February 2015, and comprised petrol pump workers. Approval was obtained from the institutional ethics committee. The sample size was statistically justified by the following equation:

$$\text{Sample Size} = (r+1(p^*)(1-p^*)(Z_\beta+Z(\alpha/2))^2 / r\,(p_1-p_2)^2$$

Where,

r=ratio of cases to controls, 1 for equal number of case and controls

p*=proportion of exposed cases + proportion of control exposed/2

Z_β=Standard normal variate for power/margin of error=for 80% power it is 0.84; for 90% it is 1.28 and or 95% it is 1.96

$Z_{\alpha/2}$=Standard normal variate for level of significance (at 5% type error it is 1.96 and at 1% type error it is 2.58

p1=proportion in cases based on previous studies

p2=proportion in controls based on previous studies

Hence, sample size = 1+ 1(0.375)(1-0.375)(1.96+1.96)2

1(0.50-0.25)2

=2 (0.375)(0.625)(15.36)

0.0625

= 115.2

This clearly indicates that that at least 115 individuals should be selected from both exposed (E) and non-exposed populations (NE).

It is important to note that buccal cell sample collection was only a part of the study, we also had to collect other biological samples such as blood as well as hair samples to get a complete scenario of their damage regarding exposure due to their occupation. As some individuals showed reluctance to such sampling, we had to cut the sample size. Buccal cells of both exposed and non-exposed subjects were collected and analysed. The blood pressure and sugar diagnostic tests were performed at the spot and persons with diabetes were ruled out of sampling procedure. People with hypertension were considered because previous studies showed that hypertension could be associated to the exposure to ingredients of the fuel and other chemicals present in the occupational settings of the workers. In the present study, only 10% workers had high blood pressure (ranging from 130/90 to 140/100) and 90% had blood pressure within the normal ranges. People exposed to X-rays or any kind of radiation a few days back, on any kind of long-term medication and cancer patients were excluded during the sample collection in both E and NE populations.

The questionnaire survey was carried out from the selected samples of both E and NE groups. The structured questionnaire was designed and one-to-one interview method was used for the survey. The questionnaire was divided into different sections of personal, occupational, medical and personal hygiene profiles of the workers. Petrol pump workers (E) were collected from the Gujrat city. The selection of petrol pumps was done randomly. Consent was obtained from the owner or managers of the pumps. For collection of buccal cell samples verbal and written permission of each worker was taken. The sampling of those workers was done who were working as fillers or dippers, i.e. having direct contact with the gasoline whereas managers were excluded from the collection criteria. Besides petrol pump workers, 100 volunteers were considered as controls (NE). These were the male respondents of more or less same socio-economic groups but never worked in petrol pumps or such settings. The NE population was working as clerks, security guards and peons in different offices and banks.

Before collection, the workers were asked to wash their mouth thoroughly. All the chemicals used were of analytical grade. The collected buccal cells were centrifuged for 10 minutes at 1500 revolutions per minute (rpm). The supernatant was then discarded. Freshly prepared peripheral blood smear (PBS) was added and centrifuged at the same speed. The procedure was repeated twice. The supernatant was discarded and the resultant semi-solid pellet was then smeared on slides and was allowed to dry in air for 8-10 minutes. After drying, fixation was done by the mixture of cold methanol and acetic acid (3:1) and then was air-dried again. After drying, Giemsa stain was used to stain the slides. After that, the slides were washed with distilled water; air-dried again and then microscopic analysis of the cells was done at 10X, 40X to count and capture each anomalies in buccal cells. Criteria assay was performed according to the method proposed by Tolbert et al.[7,8] Slides were prepared to score 3,000 cells per individual to determine the frequencies of MN, BN, KR and KL cells (Figure-1).

SPSS 16 was used for data analysis. All the data was expressed as the average and standard error of the mean (SEM). One-way analysis of variance (ANOVA) and Student's t-test was used to determine the significance of the cellular parameters. Average, standard deviation and standard error of mean was calculated for each population. Student's t-test was used for the analysis of significance of differences of mean of nuclear anomalies between the E and NE groups. The significance of the effects of dependent variables such as age, smoking habits and time duration of working (only in exposed population) with respect to increasing nuclear anomalies was determined by applying ANOVA within each exposed (E) and non-exposed groups (NE). $P \leq 0.05$ was considered significant.

Results

Of the 200 subjects, there were 100(50%) in each group. The data collected through microscopic study was compared on the basis of duration of job experience, smoking habits and age. It was observed that only 16(8%) of the petrol pump workers were using PPE. The equipment was not proper for the type of job they were performing. Only helmets were being used while the use of goggles, gloves and masks was not observed despite the fact that they were more suitable to their jobs as fillers and dippers.

In general, the exposed population revealed comparatively higher frequency of nuclear damage as compared to non-exposed ones. The frequencies of genotoxic parameters, MN and BN cells were higher among the E group as compared to the NE group (9.5±0.7, 6.4±0.5 and 1.42±0.2, 1.72±0.2), showing more genetic damaged status. Less frequency of MN as compared to BN cells within the NE group was found, showing that the population had very little genetic damage stage. The situation was the other way round in E population. The frequencies of cytotoxic parameters, KR and KL cells frequencies were higher in E group as compared to NE group (20.07±1.15, 30.93±1.56 and 5.67±0.9, 9.57±1.3), thus depicting the more toxic state of the E group.

The time duration of job experience (in years) in petrol pump workers affected the frequencies of nuclear anomalies. Job experiences on the basis of <10 and >10 years was made and it was noted that frequencies of MN, BN, KR and KL cells were higher in >10 years of experience as compared to <10 years and results were statistically significant (p=<0.05). When >10 years of job experience was split into the groups of 11-20, 21-30 and 31-40 years, a uniform increasing pattern was observed in both geno- and cytotoxic parameters, respectively (Figure-2).

Furthermore, 38(38%) participants were smokers in the exposed group compared to 27(27%) in the non-exposed group. The MN, BN KR and KL cell frequencies of exposed smokers (ES), exposed non-smokers (ENS), non-exposed smokers (NES) and non-exposed non-smokers (NENS) were calculated. Comparison was made between ES and

Table: Frequencies of cytogenetic nuclear anomalies in the buccal epithelial cells on the basis of duration of exposure, smoking habits and age of exposed (E) and non-exposed (NE) workers

Groups	Total	Age Mean±SEM	MN Mean±SEM	BN Mean±SEM	KR Mean±SEM	KL Mean±SEM
EXPOSED (E)						
Duration of exposure	<10(n=65)	-	7.5±0.5	5.4±0.6	18.08±0.8	28.8±0.7
	>10(n=35)	-	13.3±1.3*	8.3±1.0*	22±1.8*	35.2±2.3*
Smokers (ES)	n=38	38.13±2.10	12.43±2.2*a	7.7±1.59*a	21±2.04*a	32.5±2.0*a
Non-smokers (ENS)	n=62	31.50±1.39	7.7±0.8*	5.4±0.6*	19.2±1.5*	29.6±2.1*
Age	<25(n=30)	-	7.1±1.4*	5.4±1.0*	17.9±2.2*	28.2±3.0*
	>25(n=70)	-	10.5±0.8*a	6.8±0.6*a	21.8±1.3*a	32.1±1.6*a
Non-Exposed (NE)						
Smokers (NES)	n=27	38.44±2.02	4.2±0.71a	4±0.73a	11.24±1.8a	18±2.4a
Non-smokers (NENS)	n=73	30.12±1.21	0.3±0.11	0.8±0.18	3.60±0.95	6.3±1.45
Age	<25(n=35)	-	0.6±0.4	0.62±0.3	2.4±1.1	2.9±1.2
	>25(n=65)	-	1.7±0.3a	2.1±0.3a	6.9±1.2a	13.01±1.8a

*Shows significant values (P=<0.05) when compared between exposed (E) and non-exposed (NE) population
a Shows significant values (P=<0.05) when compared within their same group either exposed (E) or non-exposed (NE)
MN: Micronuclei;
BN: Binucleus;
KR: Karryorehixes;
KL: Karyolysis
SEM: Standard error of mean.

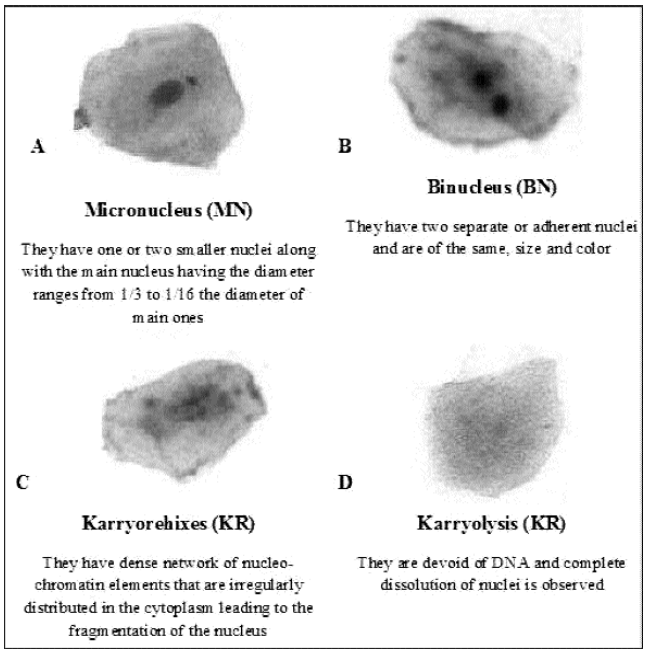
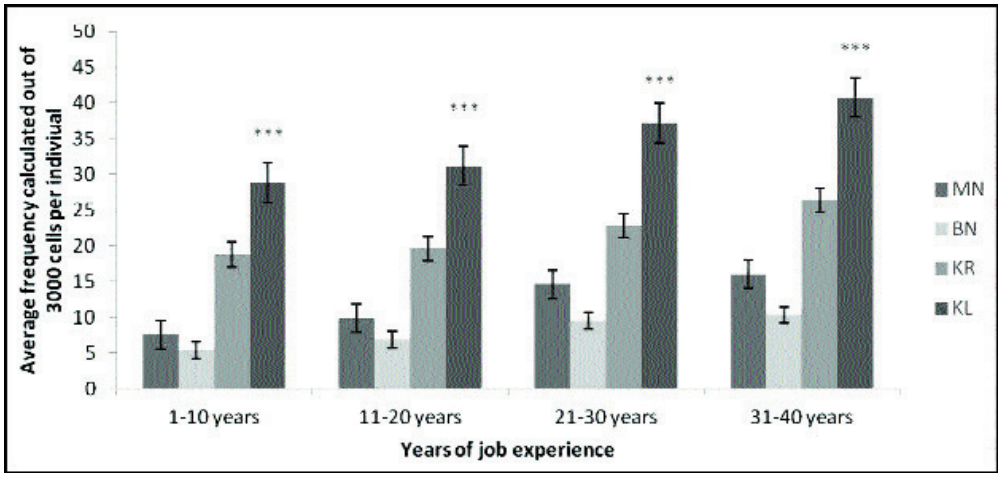


Figure-1: Photomicrographs (A-D at 40X) of different nuclear morphologies observed in the buccal epithelial cells of both exposed (E) and non-exposed (NE) populations.

ENS, ES and NES, NES and NSNE and ENS and NENS groups, respectively. Results revealed less damage in NES and NENS population, thus low incidence of genotoxic and cytotoxic cells were observed. All of the results found were statistically significant (p=<0.05).

Age-wise comparison in both E and NE population showed interesting results of more MN, BN, KR and KL cell frequencies in petrol pump workers as compared to controls. The age groups were divided into two categories of <25 and >25 years in both groups. Also, <25 years age group showed less damage as compared to >25 years within the same group and when comparison was made between the groups of E and NE population. The results calculated were statistically significant (p=<0.05) (Table).



*****Highly significant values (p=<0.001),**
MN: Micronuclei;
BN: Binucleus;
KR: Karryorehixes;
KL: Karyolysis

Figure-2: Comparison of nuclear anomalies with respect to job duration (years) in exposed population (E).

Discussion

Petrol pump workers are consistently exposed to harmful and toxic fuels and chemicals in their work environment. These exposures multiply when the workers handle such carcinogenic and mutagenic compounds without the usage of PPE. The unstable nature of petrol vapours make them easily dispersible in the atmosphere, especially at gasoline stations. Vapours of petrol are not safe even when inhaled for a very short time during fuelling vehicles which put the workers at more risk due to their occupation.[9] Workers are exposed to poisonous agents such as BaP contained in gasoline, vehicular exhaust, diesel fumes and cigarette smoke.[10] Occupational exposure to gasoline and diesel vapours has harmful effects on normal functioning of human body systems.[11]

In the present study, the average frequency of micronuclei in buccal cells was 9.8±0.7 per 3,000 cells. According to Holland N.,[12] our results are within the baseline range of 0.05-11.5 MN per 1,000 cells. Calculation of the frequencies of MN cells formed as a result of mutagenic agents is widely used for the cytogenetic bio-monitoring and predicts genomic instability.[13] The MN assay in buccal epithelial cell is easy to carry out due to its appropriateness in the laboratory and field settings. Buccal cells have little DNA repair capacity as compared to peripheral blood lymphocytes and accurately reflect the genetic instability events.[14] Age and gender are the most important factors affecting the MN index. MN's formation could be associated to: i) exogenous factors such as radiation, chemical agents and microorganism invasion; and ii) endogenous factors

including genetic defects, pathological changes, nutritional deficiencies and injuries.[15] MN cells are found in cancer cells, or in those cells that have been exposed to various risk factors.[16]

The data regarding cytogenetic toxic evaluation based on the basis of smoking habits and age of E and NE population showed that smoking plays a role of cofounding factor in the elevation of cytogenetic and cytotoxic damage. It is clear that the age of smokers from either groups was more or less similar, but more MN, BN, KR and KL cell frequencies were found in petrol pump workers. The same case was observed in the non-smokers of both groups. This reflects that the exposure of toxic compounds in gasoline and cigarette smoke that is likely to be the contributory factor in this regard. The genotoxicity as well as cytotoxicity both collectively account for the assessment of damage in the exposed population either through occupation or by smoking or alcohol intake. The cytotoxicity or cell death parameter might arise after the sudden or short-term exposure to any carcinogenic agent present in the environment and may lead to the damage of DNA in the long term when continuous exposure to toxic chemicals, either occupationally or as result of smoking or alcohol intake habits persists. Cell death can be programmed (apoptosis) or non-programmed (necrosis). KR cells are mostly associated to apoptotic form of cell death. Increase in apoptotic form of cell death after cigarette smoke extract stimulation in the alveolar cells of rat was found.[17]

The incidence of increased MN frequency in >25 years of age group as compared to <25 within the NE population indicates the fact that MN is affected by increasing age. Age-wise comparison between exposed and non-exposed population showed the similar higher trend of KR and KL in both groups. The cytotoxic parameters of exposed population showed elevated frequencies as compared to the non-exposed population, which could possibly be associated to apoptotic or necrotic form of cell death due to increasing age and exposure to carcinogenic chemicals in petrol pump workers. The feature of both forms of cell death may occur side by side in the same cell population. In response to a large number of cytotoxic agents, both cell death forms are detected. The possibility that the extent to which this continuum occurs may vary as function of increasing age.[18] Similar results were observed by several researchers[19-22] regarding petrol pump workers where nuclear anomalies were observed regarding duration of exposures, different lifestyle habits, such as smoking and alcohol intake, and increasing age as compared to controls.

The present study found that increasing age is a factor that could be related to increasing nuclear anomalies in both E and NE groups. Smoking is an additional aspect that is relevant to escalating nuclear alterations in both groups and duration of exposure is a strong feature only in exposed population associated with such nuclear damage. Although this test is widely used by the researchers in various fields for the assessment of genetic damage, it is only an indicative test. It is not a reliable test and in toxicology it is a documented fact that more than one diagnostic test is compulsory in order to reveal a contributory consequence of a determinant pollutant. The applicability of this assay in humans needs to be researched and to be further clearly described in order to improve the possible specificity and sensitivity of the test. The nuclear anomalies in buccal cells as biomarkers of different genotoxic events or as indicatory tools for cancer or other degenerative diseases require further and extensive research.

A higher frequency of buccal cells with nuclear anomalies such as MN, BN, KR, and KL was observed in the exposed group as compared to control samples and this might be due to the exposure to BaP and other carcinogenic agents present in gasoline and vehicular exhaust. It is important to encourage the medical check-ups, implement and monitor the use of PPE for workers at petrol pumps. It is also necessary to educate the workers for the usage of PPE and about the toxicity of fuels to guarantee safe and healthy working environment to reduce the health risks and hazards that they may cause severe injuries and ailments and become serious health risk.

Conclusion

The micronuclei test was used as an indicator of genotoxicity elucidation. Pump workers showed higher frequencies of cytogenetic toxicity as compared to controls due to the exposure to mutagenic and carcinogenic compounds present in automotive fuels. Smoking and age has also added to the frequencies of the damage.

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