Application of Liquid-based Cytology Preparation in Micronucleus Assay of Exfoliated Buccal Epithelial Cells in Road Construction Workers

Abstract

Background: Asphalts are bitumens that consist of complex of hydrocarbon mixtures and it is used mainly in road construction and maintenance. Aim: This study was undertaken to evaluate the micronucleus (MN) assay of exfoliated buccal epithelial cells in road construction workers using liquid-based cytology (LBC) preparation. Materials and Methods: Three different stains (May–Grunwald Giemsa, hematoxylin and eosin, and Papanicolaou) were used to evaluate the frequency of MN in exfoliated buccal epithelial of 100 participants (fifty road construction workers and fifty administrative staff) using LBC preparation. Statistical analysis was performed with Student’s t-test, and P < 0.05 was considered statistically significant. Results: The mean frequency of MN for cases was significantly higher than that of controls (P = 0.001) regardless of staining method used and also cases with exposure period of more than 5 years had statistically significant difference (P < 0.05) than cases with <5 years of exposure. Conclusion: The present study concluded that workers exposed to asphalts during road construction exhibit a higher frequency of MN in exfoliated buccal epithelial cells and they are under the significant risk of cytogenetic damage. LBC preparation has potential application for the evaluation of frequency of MN. This technique may be advocated in those who are occupationally exposed to potentially carcinogenic agents in view of improvement in the smear quality and visualization of cell morphology.

Keywords: Buccal epithelial cells, liquid-based cytology, micronucleus, road construction workers

Introduction

Micronuclei (MN) are small chromatin bodies formed by condensation of acrocentric chromosomal fragments or by whole chromosomes, lagging behind the cell division. It is present in the cytoplasm as oval or round mass. MN is the only biomarker that allows simultaneous evaluation of clastogenic and aneugenic effects in multiple cells, easily detected in interphase. MN assay in exfoliated buccal cells is a useful and minimally invasive method for monitoring genetic damage in humans in comparison to obtaining blood samples for lymphocyte and erythrocyte assays or tissue biopsies.1

Asphalts are bitumens which are a complex polycyclic aromatic hydrocarbon (PAH) mixtures and it is used mainly in road construction and maintenance. The workers engaged in this occupation are chronically exposed to coal-tar, bitumen, and asphalt fumes by inhalation and dermal absorption. The organic compounds found in asphalt fumes have been shown to be mutagenic/carcinogenic.2,3 DNA damage in the form of sister-chromatid exchange, MN, and strand breaks in asphalt workers has been recently studied, and results showed higher levels of genetic damage among exposed workers compared to controls.4,5 DNA adducts are formed when reactive metabolites bind to sites present in the DNA molecule, thus providing a useful measure of DNA damage which has been found to be associated with both PAH exposure and cancer risk.6,7 A recent study by McClean et al. observed that adduct burden was higher among asphalt workers and associated with DNA damage.8 Buccal epithelial cells form the first barrier for the inhalation or ingestion route and are capable of metabolizing carcinogens to reactive products. About 90% of human cancers originate from epithelial cells. Hence, they represent a preferred target site for early genotoxic events induced by carcinogenic agents entering the body through inhalation and ingestion.9

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Liquid-based cytology (LBC) was initially applied for the screening of cervical cancer. It offers many advantages over conventional smears; minimizes issues related to sampling, aid in preparation of high cellularity smears with homogeneous thin layer, reduction in false-negative rates, and clear background; and enhances sensitivity and quality of smears.[10] The present study was undertaken to evaluate frequency of MN of exfoliated buccal epithelial cells using LBC preparation in road construction workers.

Materials and Methods

Study population

The study population of this prospective study comprised 100 healthy participants devoid of any types of oral lesions, of whom fifty males were road construction workers (cases) and other fifty males were normal healthy individuals with no occupational exposure to toxic agents (controls). The study was conducted during the period from August 2015 to October 2015, after obtaining approval from the Institutional Ethical Committee. Informed written consent was obtained from each patient. All the participants were asked to complete the questionnaire to obtain demographic data such as age, history of smoking/alcohol, working period in building construction, family history of cancer, and general status of health.

Collection of specimens and staining

Prior collections of exfoliated buccal epithelial cells, all the participants were advised to rinse their mouth thoroughly with water to remove unwanted debris. Sterile wooden spatula was introduced into the mouth, scraped on the buccal mucosa, and material was placed in preservative solution for minimum of ½ h. The material was centrifuged at 1500 rpm for 5 min. The supernatant was discarded and pellet was agitated to get homogenous sample. One drop of normal saline was added to pellet and it was mixed well. Fifty microliter of diluted pellet was placed on clean slides with a drop of fixative solution and subsequently stained with May–Grunwald Giemsa (MGG), hematoxylin and eosin (H and E), and Papanicolaou stain (Pap). The slides were examined under light microscope by pathologist.

Evaluation criteria

According to the criteria established by Tolbert et al.,[11] and El-Setouhy et al.,[12] 1000 exfoliated buccal epithelial cells with MN, were counted per participant. Criteria of Tolbert et al. used for both choosing of cells and for identification of cells; the following parameters for cell inclusion in the cells to be scored: (a) intact cytoplasm and a relatively flat cell position on the slide, (b) little or no overlap with adjacent cells, (c) little or no debris; and (d) normal, intact smooth, and distinct nuclear perimeter; the criteria used to identify the MN: smooth and round perimeter, size less than one-third of the main nucleus diameter but large enough to discern shape and color, texture and intensity of the staining similar to that of the nucleus, same focal plane as the nucleus, and no overlap or connection to the nucleus. Criteria of El-Setouhy et al. for identification of MN: opaque extranuclear intracytoplasmic bodies viewed under an oil immersion lens and by phase contrast. However, binucleate cells or cells with fragmented nuclei, karyolysis, and nuclei appearing as broken eggs were not counted as MN.

Statistical analysis

All the statistical analyses were performed using Statistical Package for the Social Sciences Statistics for Windows (version 20.0. Armonk, New York: IBM Corporation, NY, USA). Master charts were prepared for frequency of MN per 1000 cells. The mean, median, standard deviation, and minimum and maximum values were calculated. The mean frequencies of MN cases and controls were compared for all the stains with Student’s t-test. Values for \( P < 0.05 \) were considered statistically significant.

Results

All the participants in both cases and controls were male, nonsmokers, and not tobacco chewers. The age range of cases and controls was 24–60 and 25–58 years, respectively. The duration of exposure ranged from 1 to 35 years. Demographic characteristics of the both cases and controls are presented in Table 1. In total, 2809 MN were counted; of these, 800 MN were with MGG, 890 were with H and E, and 1119 were with Pap stain. The mean frequency of MN stained with MGG [Figure 1], H and E [Figure 2], and Pap stain [Figure 3] for cases and controls was 16 versus 7.66, 17.8 versus 8.96, and 22.38 versus 13.56, respectively, and it was statistically significant (\( P = 0.001 \)) regardless of staining method used [Table 2]. The effect of age (<30 and >30 years) and alcohol consumption on the frequency of MN were also evaluated in both controls and cases and did not detect any significant differences. However, cases with exposure period of more than 5 years had statistically significant difference than cases with <5 years of exposure [Tables 3 and 4].

Discussion

Higher frequencies of MN in exfoliated buccal epithelial cells were observed in those who are occupationally exposed to toxic environmental agents such as solvents, PAHs, emissions of sugarcane straw burning, gasoline,
arsenic, and antineoplastic drugs compared to the controls.\cite{9} Due to its ease of use and handling, low capital investment requirements, and the accuracy achieved, MN assay in exfoliated buccal epithelial cells has received special attention to assess the impact of environmental and genetic factors and lifestyle habits on genomic instability in humans.\cite{13,14} Hence, buccal epithelium cells provide an alternative source of tissue in human subjects monitoring for occupational and environmental toxic exposures.\cite{15}

Increased risks of the lung, stomach, bladder, leukemia, and nonmelanoma skin cancer are seen in individuals exposed to asphalt mixture.\cite{8}

The present study evaluated the genotoxicity in the road construction workers and found that they had higher frequencies of MN compared to that of controls, regardless of staining method used. These findings were considered statistically significant ($P = 0.001$). Similar to the current study, Çelik et al.\cite{16} conducted a study on biomonitoring for the genotoxic assessment in road construction workers in buccal epithelial cells and found statistically significant increase in the frequency of MN-exposed group compared with control group ($P < 0.001$). Burgaz et al.\cite{5} conducted a work on cytogenetic biomonitoring of workers exposed to bitumen fumes and concluded that road paving workers had significant cytogenetic damage in peripheral blood lymphocytes compared to controls ($P < 0.0001$).

A variety of staining methods employed for the staining of MN in exfoliated buccal epithelial cells, namely Feulgen/fast Green, fluorescent dyes includes 4’,6-diamidino-2-phenylindole, acridine orange, Hoechst and propidium iodide, Pap, and MGG stains.\cite{17} Bonassi et al.\cite{18} conducted a survey and found that over 50% of the participating laboratories used Feulgen/fast Green stain. This method stains only nuclear fragments but not cytoplasm due to its DNA specificity, hence it is widely used for MN assay. About 30% of the studies utilized DNA nonspecific stains such as MGG and Pap.\cite{9} In the present study, slides were stained with three different stains (MGG, H and E, and Pap) using LBC preparation in both groups and observed greater frequencies of MN in contrast to study done by Çelik et al.,\cite{16} who observed less number of MN using Feulgen/fast Green stain. Similar findings were observed in Grover et al.,\cite{19} who compared Feulgen/fast Green and Pap stains, and in Nersesyan et al.,\cite{20} Feulgen/fast Green and Giemsa were compared.

Epidemiological studies show a strong association between frequency of MN and variety of factors such as environmental and occupational exposures, radiotherapy, chemotherapy, lifestyle habits, oral, and other cancers.\cite{21} On the basis of questionnaire and general examination, possibilities of other occupational and environmental exposures, radiotherapy, chemotherapy, oral, and other cancers causing increased frequencies of MN were excluded from the study. Lifestyle factors include smoking, alcohol consumption, and tobacco chewing can also lead to higher MN frequency. A study done by Motgi

### Table 2: Comparison of mean frequency of micronucleus using Student’s $t$-test among all stains

| Stain   | Cases  | Controls | $P^*$ |
|---------|--------|----------|-------|
|         | $n$    | Mean±SD  | Median | Minimum–maximum values | $n$    | Mean±SD  | Median | Minimum–maximum values |       |
| MGG     | 50     | 16±5     | 16     | 7-42          | 50     | 7.66±4.77 | 7      | 4-38          | 0.001 |
| H and E | 50     | 17.8±5.67| 18.5   | 4-48          | 50     | 8.96±4.96 | 8      | 6-40          | 0.001 |
| Pap     | 50     | 22.38±4.79| 21.5  | 15-47         | 50     | 13.56±5.28| 12.5   | 6-43          | 0.001 |

* $P<0.05$ was considered statistically significant. MGG=May–Grunwald Giemsa, H and E=Hematoxylin and eosin, Pap=Papanicolaou stain, MN=Micronucleus, SD=Standard deviation.
et al.\cite{2} concluded that uses of smokeless and smoked tobacco are associated with cytotoxic and genotoxic effects. Workers observed an increase in frequencies of MN with the consumption of alcohol in individuals who have genetic variants in the alcohol-metabolizing enzyme, alcohol dehydrogenase.\cite{21} In this study, smokers and tobacco chewers were not included in the study, but 28% in cases and 26% in controls were alcoholics. However, no statistically significant difference was found between alcohol intake and MN frequency. Hence, asphalt or bitumens were responsible for increased frequency of MN in the present study.

Kujan et al.\cite{23} first applied LBC in exfoliated buccal epithelial cells and found a uniform distribution of cells in addition to improved cytomorphology and visibility of nuclear details. A study done by Hayama et al.\cite{24} showed a statistically significant improvement in smear thickness, cell distribution, and a reduction in cell overlapping and the presence of blood.

In the present study, slides prepared using LBC technique were good quality with clean background with minimum mucus, erythrocytes, inflammatory cells, and microbial colonies. Our study also showed single layer of cells, adequate cellularity, and improved cell morphology. Hence, this preparation reduces the likelihood of false-negative results. The residual sample can be used for advanced procedure like immunocytochemistry. Thus, application of LBC in MN assay is one way to refine and improve the assessment of this biomarker.

Even though evaluation of MN is a sensitive, noninvasive, and low-cost method to detect cytogenetic damage, it has some limitations. The present study was done using

### Table 3: Frequency of micronucleus with respect to age and alcohol consumption in controls among various stains (n=50)

| Stain       | Individuals | MN (mean±SD) | P* |
|-------------|-------------|--------------|----|
| MGG         | Age (year)  |              |    |
| <30 (n=17)  | 7.46±4.54   | 0.93         |    |
| >30 (n=33)  | 7.58±4.62   | 0.95         |    |
| Alcohol consumption | Yes (n=13) | 7.54±4.47 | 0.95 | |
| No (n=37)   | 7.45±4.23   | 0.95         |    |
| H and E     | Age (year)  |              |    |
| <30 (n=17)  | 8.86±4.79   | 0.97         |    |
| >30 (n=33)  | 8.90±4.64   | 0.92         |    |
| Alcohol consumption | Yes (n=13) | 8.90±4.91 | 0.92 | |
| No (n=37)   | 8.74±4.88   | 0.92         |    |
| Pap         | Age (year)  |              |    |
| <30 (n=17)  | 13.47±5.11  | 0.97         |    |
| >30 (n=33)  | 13.52±5.21  | 0.83         |    |
| Alcohol consumption | Yes (n=13) | 13.51±5.12 | 0.83 | |
| No (n=37)   | 13.16±5.23  | 0.83         |    |

*P<0.05 was considered statistically significant.

MGG=May–Grunwald Giemsa, H and E=Hematoxylin and eosin, Pap=Papanicolaou stain, SD=Standard deviation

### Table 4: Frequency of micronucleus with respect to age, alcohol consumption, and years of working exposure in cases among various stains (n=50)

| Stain       | Individuals | MN (mean±SD) | P* |
|-------------|-------------|--------------|----|
| MGG         | Age (year)  |              |    |
| <30 (n=23)  | 14.87±4.91  | 0.46         |    |
| >30 (n=27)  | 15.89±4.94  | 0.07         |    |
| Alcohol consumption | Yes (n=14)  | 15.94±4.98   | 0.07 |
| No (n=36)   | 13.01±4.75  | 0.07         |    |
| Working exposure (year) | <5 (n=21) | 11.84±4.21 | 0.002 | |
| >5 (n=29)   | 15.98±4.84  | 0.002        |    |
| H and E     | Age (year)  |              |    |
| <30 (n=23)  | 16.52±5.26  | 0.48         |    |
| >30 (n=27)  | 17.6±5.61   | 0.07         |    |
| Alcohol consumption | Yes (n=14)  | 17.7±5.58   | 0.07 |
| No (n=36)   | 14.42±5.32  | 0.07         |    |
| Working exposure (year) | <5 (n=21) | 12.97±4.76 | 0.003 | |
| >5 (n=29)   | 17.5±5.59   | 0.003        |    |
| Pap         | Age (year)  |              |    |
| <30 (n=23)  | 21.42±4.17  | 0.49         |    |
| >30 (n=27)  | 22.26±4.49  | 0.49         |    |
| Alcohol consumption | Yes (n=14)  | 21.15±4.58  | 0.07 |
| No (n=36)   | 18.5±4.36   | 0.07         |    |
| Working exposure (year) | <5 (n=21) | 18.13±4.34 | 0.002 | |
| >5 (n=29)   | 22.31±4.69  | 0.002        |    |

*P<0.05 was considered statistically significant. MGG=May–Grunwald Giemsa, H and E=Hematoxylin and eosin, Pap=Papanicolaou stain, SD=Standard deviation
DNA nonspecific stains; hence, study with DNA-specific stain would have increased the specificity. Since sample size was small, still large scale work should be done for longer duration with follow-up for the confirmation of results obtained in this study. The current study evaluated the period of exposure of asphalts to cases. However, rate of exposure of was not assessed and this could be another limitation of study.

**Conclusion**

The present study concluded that workers exposed to asphalts during road construction exhibit a higher frequency of MN in exfoliated buccal epithelial cells and they are under the significant risk of cytogenetic damage. LBC preparation has potential application for the evaluation of frequency of MN. This technique may be advocated in those who are occupationally exposed to potentially carcinogenic agents in view of improvement in the smear quality and visualization of cell morphology.

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Nil.

**Conflicts of interest**

There are no conflicts of interest.

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