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Type of Chemical Exposition Causes Different Nuclear Abnormality Frequencies

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ABSTRACT

The systematic epidemiological monitoring of populations exposed to chemical agents, is one type of sanitary control that allows early detection of health problems. Human biomonitoring may be conducted with various cytogenetic tests, which evaluate genotoxic effects by detecting DNA damage. One validated and accepted test is the Micronucleus Assay, applied to the buccal epithelial cells. Workers, who were occupationally exposed, participated in our survey: 60 sugar cane cutters, 30 gasoline attendants, and 30 pesticide-sprayer pilots of small airplanes. Comparing the three groups, sugar cane cutters revealed higher fold increase (FI) of abnormalities: pyknotic nuclei (FI=31.4), chromatin condensation (FI=18.8) and binucleated cells (FI=15.0); gasoline attendants exhibited chromatin condensation (FI=14.5) and micronucleus (FI=3.6), and pilots showed severe nuclear abnormalities, counted as binucleated cells (FI=269.5), pyknotic nuclei (FI=54.2) and cells with chromatin condensation (FI=45.2). The biomonitoring of the side effects derived of exposure to genotoxic substances, in the workplace, such as the one we have done for this research, is fundamental for the design of corrective and preventive strategies that assure the health of the worker and biosecure working conditions. Detection of these biomarkers in exposed people indicates genotoxicity events and increased risk of disease development.

1. Introduction

The biomarkers used in monitoring studies can be defined as indicators and predictors of mechanisms, events, or changes that take place in biological systems. They are used as evidence of exposure, to determine the effects of the toxic substances and to measure genetic susceptibility, allowing to diagnose environmentally induced adverse outcomes in humans [1]. DNA damage biomarkers act as specific indicators of environmental exposures, and allow to predict consequences in people exposed daily to a variety of genotoxic insults. The selection of the most appropriate samples to determine DNA damage, should consider the characteristics of exposed population, exposure type and involved pollutants, endpoint mechanisms, and persistence of DNA damage, rate of cell turnover, sample timing, tissue accessibility, study aims, as well as possible ethical and social restrictions [1].

DNA damage is involved in the development and progression of many diseases. Severe exposures to genotoxic substances may occur in certain occupations and lead to changes in mechanisms of chromosomal division. Effects of these exposures can be evaluated through epidemiological studies using appropriate biomarkers [2, 3]. One of such biomarkers of abnormal cell division, involves chromosomal breakage, miss-segregated chromatin or mitotic interferences observed as nuclear abnormalities. In this sense, the frequency of nuclear abnormalities that appear in the epithelial cells, as cytotoxic effect, may reflect possible health status in people exposed to genotoxic and carcinogenic agents [2]. To fulfill the subject, it is essential to use a minimally invasive method for the determination of these biomarkers. For this purpose, micronucleus and other nuclear abnormalities assays in exfoliated oral cells, are excellent tools for biomonitoring of environmental or occupational exposures to genotoxic substances [4-8]. The collection of oral cells is the least invasive method applicable for measuring DNA damage in humans, comparing to blood samples used in lymphocyte and erythrocyte assays, or tissue biopsies in biomonitoring of environmental or occupational exposures to genotoxic substances [4-8].

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It is known that carcinogenesis occurs as a result of the accumulation of genetic events, which can be detected cytogenetically. Therefore, assessment of these effects in populations that are occupationally exposed to genotoxic substances or potential carcinogens should be an important approach in preventive medicine. Hence, we compared DNA damage results, expressed by the frequencies of nuclear abnormalities in exfoliated buccal epithelial cells, measured among three populations previously monitored and occupationally exposed to three different potentially carcinogenic substances, such as: ashes of burned sugar cane, gasoline and pesticides vapors [6-8].

2. Experimental Methods

2.1 Participants

In this study, all participants were randomly selected and they signed an informed consent. The protocol was approved by the Ethics Committee of Universidad de Occidente.

The first group was formed by 60 sugar cane cutters from Sinaloa, Mexico. These workers have been cutting sugar cane, for at least the last three consecutive years, and exposed to their ashes, derived from the cane harvest process. Sugar cane ashes are composed principally by six polycyclic aromatic hydrocarbons (PAHs): acenaphthylene, fluorene, retene, 2-methylanthracene, fluoranthene and pyrene [9] and are considered carcinogenic and mutagenic for humans, according to the EPA pollutant list. Sixty unexposed, administrative workers were considered as control group [6].

The second study group was constituted by gasoline station attendants from Los Mochis City, Sinaloa, Mexico, formed by 60 gasoline attendants, exposed to gasoline vapors. Gasoline is a blended mixture of hydrocarbons formed primarily by paraffins, cycloparaffins and aromatic and olefin hydrocarbons with carbon numbers greater than C3 and boiling in the range of 30–260 °C [10]. As control group, were considered 60 individuals not exposed to gasoline vapors [8].

The third study group was formed by 30 male pilots of small airplanes that aerially sprayed pesticide on fields at Sinaloa, Mexico, and 30 male control unexposed individuals, who have no history of exposure to pesticides. The pilots were exposed to a mixture of pesticides used

habitually for crop protection in fields in Sinaloa, Mexico [8]. All participants were asked to rinse their mouth with water and donate the epithelial cells sample of the oral mucosa by scraping the inner walls of the cheeks with wooden spatula.

2.2 Staining Procedure

The epithelial cell samples were placed on a slide, dried in air, fixed with methanol–acetic acid (3:1) and stained with the Feulgen reaction. To avoid bias in counting, all slides were coded. Microscopic counts of buccal cells were performed according to the typical shape and size that defined nucleus and cytoplasm [11]. Genetic abnormalities were counted and sustained on morphology and localization of nuclear abnormalities in the cell in agreement to the procedure of Stich and Rosin [12] and Martinez-Valenzuela et al. [13]. They were classified and counted conforming to Tolbert et al. [14] and Thomas et al. [15] determining the frequency of micronucleus and other nuclear abnormalities such as pyknosis, chromatin condensation, nuclear buds, karyolisis and binucleated cells. Estimation of the abnormal cell frequencies was performed according to the criteria of Stich [16].

2.3 Statistical Data Analysis

Statistical analysis was conducted using the statistical software Minitab 15; values of nuclear abnormalities were expressed as number of abnormal cells among 1000 observed cells and these were expressed as arithmetic mean ± standard errors of mean (SEM). In order to determine the degree of association between nuclear abnormalities, among the three exposed groups, the ANOVA test which compares the equality of population means, with posterior pairwise comparison of means using Hsu's MCB test was applied. Moreover, to observe the differences between nuclear abnormality results of exposed participants and controls, the Fold Increases (FI's) were calculated. They express the magnitude of differences between the mean values of nuclear abnormalities from exposed individuals divided by the values of corresponding controls [17].

3. Results and Discussion

The results of nuclear abnormality frequencies of 60 sugar cane cutters exposed to burned sugar cane plant ashes [6], 60 gasoline attendants exposed to gasoline vapors [7], and 30 pilots exposed to pesticides sprayed on farmlands [8] indicated higher frequencies of all nuclear abnormalities comparing to their respective controls (Table 1 and Fig. 1).

Table 1 Comparison of nuclear abnormalities frequencies (X±SEM) among three study groups

	Sugar cane cutters	Gasoline attendants	Pilots pesticide sprayers	ANOVA *p*
Micronucleus	4.1 ± 0.5*	6.6 ± 0.5	6.1 ± 0.4	0.000*
Pyknotic nuclei	182.4 ± 10.7	78.4 ± 9.6*	189.8 ± 25.1	0.000*
Chromatin condensation	127.9 ± 11.0	164.1 ± 16.8	366.0 ±26.8*	0.000*
Nuclear buds	10.9 ± 0.7	6.3 ± 0.8*	16.1 ± 3.3	0.000*
Karyolitic cells	17.4 ± 1.2*	3.0 ± 0.4	7.4 ± 0.8	0.000*
Binucleated cells	15.0 ± 0.8	6.5 ± 0.8	107.8 ±22.2*	0.000*

** Statistically significant value*

The ANOVA test revealed these groups, as three statistically significant different groups (p=0.000). Among studied groups, executing Hsu's MCB test, it was indicated that the lowest increase of nuclear abnormality frequencies corresponded to gasoline attendants exposed to paraffins, cycloparaffins, and aromatic and olefin hydrocarbons. Whereas, the highest increase of nuclear abnormalities were observed as binucleated cells, pyknotic cells and cells with chromatin condensation in pilots, indicating specific high cytotoxicity induced by pesticides. The sugar cane cutters exposed to ashes, which contain PAHs, revealed intermediate increases of nuclear abnormality frequencies expressed by cells with pyknotic nuclei, chromatin condensation and binucleated cells.

The results of FI's of nuclear abnormalities among studied participants compared to the corresponding controls are summarized in Table 2. The highest FI value agreed to binucleated cells (FI=269.5), cells with pyknotic nuclei (FI=54.2) and cells with chromatin condensation (FI=45.2), observed in pilots dispersing pesticides on Sinaloa farmlands. The study showed that the group of occupationally exposed workers developed nuclear abnormalities, caused by their professional exposure. The second place, in nuclear damages, was obtained by sugar cane cutters group, in which pyknotic nuclei, chromatin condensation and binucleated cells predominated among nuclear abnormalities. Gasoline attendants showed the lowest degree of nuclear abnormalities.

Table 2 Comparison of the Fold Increases between the studied groups

	Sugar cane cutters	Gasoline attendants	Pilots pesticide sprayers
Micronucleus	3.2	3.6	3.6
Pyknotic nuclei	31.4	2.7	54.2*
Chromatin condensation	18.8	14.5	45.2*
Nuclear buds	3.2	1.7	3.7
Karyolitic cells	4.6*	2.0	2.0
Binucleated cells	15.0	2.5	269.5*

**Statistically significant value*

The micronucleus assay (MN) is a mutagenic test useful for the detection of chemical agents, able to produce chromosome structure modification and disruption of segregation that induces the formation of small membrane bound DNA fragments (micronuclei in the cytoplasm of interphase cells). This assay is, *in vivo* test, to evaluate genotoxicity, clastogenicity and aneugenicity. The technique applied to oral epithelium is particularly attractive, since these cells can be collected in a minimally invasive manner. Also, MN assay has been applied to investigate, and to evaluate, the impact of human exposure to chemical agents that damage DNA [18]. The range of nuclear abnormalities associated with different types of toxic agents can be seen in Table 1.

Besides micronucleus, there are several abnormalities able to be analyzed with micronucleus assay: binucleated cells, nuclear buds, pyknotic cells, karyolitic cells and cells with condensed chromatin. It should be noted that some of these effects e.g. cells with pyknotic nuclei, condensed chromatin and binucleated have a higher frequency of occurrence than cells with micronucleus. Thereby, estimation of their statistical power should be of a specific interest to complete the evaluation of characteristic environmental exposures and their effects on DNA detriment [19].

Spontaneous mutations are unpredictable events and they are characterized by a change in the structure or sequence of a normal gene. Certain conditions or different agents may specifically increase the likelihood of DNA damage [20]. Gene amplification and genome damage are expressed by frequencies of micronucleus and nuclear buds, whereas cell death is expressed by apoptosis and karyolisis [21]. These expressions observed in oral cell systems provide a more comprehensive assessment of genome damage, than only micronucleus frequencies determined in context of cytotoxicity and cytostatic effects, originated by environmental or occupational exposures. Comparing the occupationally exposed workers, we found different scenarios. Sugar cane cutters exposed to combustion gases and ashes from burned sugar plants, which contained principally PAHs, revealed severe degree of abnormalities expressed by: pyknotic nuclei (FI=31.4), chromatin condensation (FI=18.8) and binucleated cells (FI=15.0) [6]. In the other hand, gasoline attendants exposed to hydrocarbons, such as benzene, toluene, xylene, pentane, butane, and isopentane exhibited a high degree of chromatin condensation (FI=14.5) and micronucleus (FI=3.6), compared to sugar cane cutters. The pilots, pesticide applicators that during their workday manipulated and sprayed pesticide mixtures, presented severe nuclear abnormalities counted as binucleated cells (FI=269.5), pyknotic nuclei (FI=54.2) and cells with chromatin condensation (FI=45.2).

The mechanisms responsible for nuclear abnormalities involve cell division failures, cell death processes, and genotoxicity and/or mutagenicity induced by chemical compounds. The evidences suggest that the nuclear buds formation, expressed with not statistically significant increase of frequencies, is the mechanism by which cells remove amplified DNA, marking gene amplification. A strong correlation exists between micronucleus and nuclear buds formation, process associated with genomic instability after exposure to different genotoxic chemicals. The micronucleus FI's were significantly increased among exposed workers versus controls, being higher than threefold. The comparison between the three groups exposed did not reveal significant differences between them. However, the nuclear buds lowest frequency (FI=1.7) was observed in gasoline attendants, while the sugar cane cutters (FI=3.2) and pilots (FI=3.7) showed higher rates.

Chromosomal damage in epithelial cells was observed as nuclear abnormalities, especially binucleated cells. This finding was very frequent in pilots (FI=269.5) and less frequently observed in gasoline attendants (FI=2.5); the highest FI (FI=45.2) of cells with chromatin condensation was observed in pilots and the lowest (FI=14.5), corresponded to gasoline attendants; a FI=54.2 for pyknotic cells was found in pilots, while gas station workers showed the lowest FI (2.7). Karyolytic cells were observed more frequently (FI=4.6) in sugar cane cutters, which contrasted with a FI = 2.0 of pilots and gas station workers.

Karyolysis and pyknosis are associated with cytotoxicity (necrosis and keratinization) and genotoxicity accompanies the early stages of apoptosis, considering these nuclear abnormalities as effective biomarker

for populations exposed to mutagenic and carcinogenic substances [18, 22-24]. According to Holland et al. [5], the observation in oral epithelial samples, of micronuclei and other nuclear abnormalities, provide a more comprehensive assessment of chromosomal damage, particularly considering cytotoxic effects of exposures, as is shown in Table 2. Hence, micronucleus assay of exfoliated cells can be considered as a preferential method to evaluate the changes referred and as a sensitive and non-invasive method for monitoring DNA damage in human populations [25].

Cells with condensed chromatin was observed with the highest frequency in pilots (FI=45.2), and this change is characterized by a nucleus with a striated pattern, due to parallel areas of condensed chromatin that are intensively stained and which has little or no transcriptional activity. Presence of these cells in oral epithelial smears has been associated with apoptosis that causes nuclear fragmentation and generation of nuclear bodies. Pyknotic cells were observed more frequently among pilots exposed to pesticides (FI=54.2) and sugar cane cutters (FI=31.4), exposed to burned plant ashes. Pyknotic cells are terminally differentiated cells that have a small shrunken, intensively stained nucleus. The nuclear diameter is usually one to two-thirds of a nucleus in normal terminally differentiated cells. Pyknosis represents an irreversible condensation of chromatin in a nucleus of cells undergoing apoptosis. The frequency of pyknotic cells in our studies correlated positively with the frequency of cells with condensed chromatin; the proportion of frequencies of condensed chromatin to pyknosis among the three populations were as follows: sugar cane cutters 0.70, gasoline attendants 2.09, and pesticide sprayers 1.93; the calculation of FI's proportion for these groups was: 0.60 (sugar cane cutters), 5.37 (gasoline attendants) and 0.83 (pesticide sprayers), suggesting that the cells that are in process of dying caused by occupational exposure, are more frequently observed in gasoline attendants. Binucleated cells were more frequent in pilots (FI=269.5) and sugar cane cutters (FI=15.0). Binucleated cells had two nuclei with the same size, morphology, texture and staining intensity. They were often very close to each other, or could even be in contact. The most likely mechanism for the binucleated cells creation, is the failure of cytokinesis either due to defects in development of the microfilament ring or cell cycle arrest caused by inadequate segregation of chromosomes or telomere dysfunction. It has been shown that non-disjunction occurs with a higher frequency in binucleated cells that fail to complete cytokinesis, rather than in cells that have completed cytokinesis, resulting in the normal formation of two mononucleated cells. Furthermore, it was observed that human epithelial cells that exhibit telomere dysfunction are more likely to become tetraploid cells following failed cytokinesis as a result of the perseverance of anaphase bridges within the cleavage plane. Individuals characterized by abnormal rate of aneuploidy, such as Down's syndrome patients [26, 27], showed a twofold higher frequency of binucleated cells compared to normal matched controls, whereas in the three worker groups analyzed here, FI's for pilots reached 269.5; for sugar cane cutters was 15.0 and for gasoline attendants was 2.5. Hence, the binucleated cell frequencies express cytokinesis failure and susceptibility to aneuploidy.

Karyolitic cells were observed very frequently in sugar cane cutters (FI=4.6) while gasoline attendants and pilots pesticide sprayers showed FI=2.0. Karyolisis is the stage when disintegration of the nucleus is complete and occurs in the later stages of necrosis and apoptosis. Whereas, cells with micronuclei which frequencies in our studies not differs significantly among three worker groups (FI= 3.2, 3.6, 3.6) are characterized by the presence of one or more micronuclei beside the main nucleus (mononucleated cells) or nuclei (binucleated cells). Micronuclei are usually observed and scored in normal transitional or terminally differentiated cells but they may also occur sometimes in basal cells. Oral cells with multiple micronuclei are rare in healthy subjects, but they may be present in the mucosa of subjects exposed to genotoxic substances. Cells with nuclear buds observed with minor frequencies among gasoline attenders (FI=1.7) compared to pilots (FI=3.7) and sugar cane cutters (FI=3.2) contained nuclear bodies that were connected to the main nucleus by a wide or a thin nucleoplasmic bridge. Nuclear buds have the same texture and stain intensity as the main nucleus. Their structure is suggestive of a budding process involved in the elimination of excess nuclear material, such as unresolved DNA repair complexes or amplified DNA following its segregation to the periphery of the nucleus.

Aneuploidy is an integral factor in the development of malignancies reflected by DNA abnormalities that change in frequency, have a predictive value and serves as a cancer risk biomarker, providing additional information on aneugenic agent's action [28]. The research opportunities afforded by oral cells, permits that regulatory mechanisms, signaling pathways and genetic modulation can be assessed before, during, and following chemical exposure. Therefore, oral cells not only offer the opportunities for early clinical diagnosis, but also to provide a unique model for mutation research, correlating genetic alterations with histopathologic changes [29].

Casartelli et al. [30] observed micronucleus frequencies in exfoliated oral cells in normal mucosa, precancerous lesions and squamous cell carcinoma. They concluded that the gradual increase in micronucleus counts from normal mucosal to precancerous lesions and to carcinoma implied a link of this biomarker with neoplastic progression.

Epidemiological data have shown increased genotoxic effects secondary to exposure to environmental mutagens that is reflected in the referred nuclear abnormalities [19]. The kinetics of replication and half-life of oral cells may affect micronucleus expression, but these differences become unimportant in chronic exposures, phenomena that we viewed in our studies analyzing nuclear abnormality frequencies of occupationally exposed workers during their workday [6-8]. This is because chronic exposure leads to a steady-state of elevated expression level of nuclear abnormalities regardless of division rate if the period of exposure exceeds the time frame for one nuclear division [31]. Hence, nuclear studies that evaluate chromosomal instability, commonly observed in diseases such as cancer, can assess the potential risk of cancer in occupationally exposed populations, such as those of our survey workers. Higher frequencies of these abnormalities have been observed in exfoliated oral cells from the occupationally exposed groups analyzed here, compared to the unexposed corresponding controls. It is known that carcinogenesis occurs as a result of the accumulation of genetic events that can be detected cytogenetically. This type of study is an important tool in preventive medicine [32, 33].

Our previous studies [6-8] demonstrated that nuclear abnormalities are useful indicators of chemical exposures and also, of different toxic responses, increasing the sensitivity of the oral epithelial cell assessment to genotoxicity. Increased abnormal cell frequencies in the three worker groups, might be due to chemical induced genetic damage, originated by diverse biotransformation mechanisms. Exposure to volatile hydrocarbons and pesticides could invariably modify the individual capacity to metabolize xenobiotics by the decreased activity of detoxifying enzymes. If the xenobiotic is not detoxified, its metabolism would yield products, which activate pathways of cytotoxicity and genotoxicity [34]. On the other hand, analysis of oral cells of workers from Sinaloa, Mexico revealed changes in nuclear abnormality frequencies, indicating that the exposure levels encountered have induced detectable clastogenic or aneugenic effects in the studied workers. Our study allowed to observe that exposure to different chemical agents caused distinct nuclear responses. The highest nuclear abnormality frequencies corresponded to the pilots, exposed to pesticide vapors and the lowest one to gasoline attendants that inhaled gasoline vapors.

Investigations on nuclear abnormality frequencies support the widely accepted assumption that they are a product of early events in human carcinogenic processes, especially because they are virtually absent in unexposed mucosa samples. The assay in oral exfoliated cells can be used as a simple reliable marker to assess the genotoxicity and for the early diagnosis of premalignant and malignant lesions and as an indicator of genotoxic damage in exposed persons [35].

4. Conclusion

The major advantage of DNA abnormality assay is its efficiency as a reliable measure of genetic instability. The assay in human exfoliated cells from the oral cavity is a minimally invasive and sensitive method used for measuring DNA damage in human populations, and to monitor nuclear abnormalities in individuals exposed to genotoxic agents.

Our results made it clear that occupationally exposed workers showed characteristic increases of cell frequencies with nuclear abnormalities, due to the specific genotoxic effects of the pollutants to which they were exposed. In general, all the assessments and biomarkers which revealed differences in nuclear abnormalities, between unexposed controls and exposed workers, suggested a probable linkage between the class of exposition to pollutants and the type of nuclear abnormalities detected.

Extensive studies to evaluate biological damage, especially to DNA, are recommended to public agencies concerned with environmental quality, occupational exposition and public health. Mutagenic investigation is one of the necessary evaluations to ensure environmental quality and occupational health, as well as the worker's education about genetic damage originated by occupational exposure, and the consequential risk for serious diseases (Benites et al. 2006). Our results stated divergence in effects of occupational exposures on nuclear abnormalities, and may provide a starting point for defining assessment groups of persons that reveal a cumulative health risk effects expressed by the magnitude of nuclear abnormalities in oral epithelial cells.

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