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Biomonitoring of oral epithelial cells in petrol station attendants: Comparison between buccal mucosa and lateral border of the tongue

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ABSTRACT

Owing to the influence of geno- and cytotoxicity on chemical carcinogenesis, studies have demonstrated that petroleum derivatives are able to induce genetic damage and cellular death with conflicting results so far. The aim of the present study was to comparatively evaluate DNA damage (micronucleus) and cellular death (pyknosis, karyolysis and karyorrhexis) in exfoliated oral mucosa cells from gas petrol attendants using two different anatomic buccal sites: cheek mucosa and lateral border of the tongue. A total of 23 gas petrol attendants and 23 health controls (non-exposed individuals) were included in this setting. Individuals had epithelial cells from cheek and lateral border of the tongue mechanically exfoliated, placed in fixative and dropped in clean slides which were checked for the above nuclear phenotypes. The results pointed out significant statistical differences ($p<0.05$) of micronucleated oral mucosa cells from gas petrol attendants for both oral sites evaluated. In the same way, petroleum derivate exposure was able to increase other nuclear alterations closely related to cytotoxicity such as karyorrhexis, pyknosis and karyolysis, being the most pronunciated effects as those found in the lateral border of the tongue. No interaction was observed between smoking and petroleum exposure. In summary, these data indicate that gas petrol attendants comprise a high risk group for DNA damage and cellular death. It seems that the lateral border of the tongue is a more sensitive site to geno- and cytotoxic insult induced by petroleum derivates.

1. Introduction

Chemical carcinogenesis is a complex multistage process that includes three main steps: initiation, promotion, and progression (Ribeiro et al., 2004). Initiation involves mainly intracellular events, which trigger irreversible changes in the cell genotype and determine its malignant transformation; the promotion stage includes processes responsible for the formation of the malignant phenotype and survival of tumor cells (Belitsky and Yakubovskaya, 2008). Progression consists of production and selection of cell clones able to compete favorably with the normal cell environment (Belitsky and Yakubovskaya, 2008). With the increasing knowledge of these mechanisms, and the conclusion that most cases of cancer are preventable, efforts have focused on identifying the agents and exposures that cause cancer (Ribeiro, 2008).

Accumulating evidence suggests that petroleum derivate compounds constitute a complex mixture of chemicals, some of them well-known genotoxic agents (Pitarque et al., 1997). According to the International Agency for Research on Cancer (IARC), the exposure to gasoline vapors is classified as carcinogenic to humans, especially on the basis of the well-established carcinogenicity of some components such as benzene (Carere et al., 1995). Particularly, petrol station attendants are workers chronically exposed to petroleum derivatives primarily through inhalation and aspiration of the volatile fraction of petrol during vehicle refueling. Therefore, risk assessment represents relevant data for protecting these individuals against potential harm, such as cancer in different target tissues, especially because there are few data in the literature with conflicting results so far (Celik et al., 2003).

Biomarkers have been used in medicine and toxicology for many years to assist in diagnosing, staging disease as well as evaluating the risk assessment. They should allow statements concerning environmental exposure and further give information on the status of susceptibility. Biomarkers are divided into three groups: the first to define the exposure to carcinogenic agents, the second to show biological effects on the target tissue and the third to give information about the individual susceptibility (Induslki and Lutz, 1997). To date, a variety of assays has been proposed as potential biomarkers in biomonitoring studies, including those that assess metaphase chromosomal aberrations, sister chromatid exchanges and host cell reactivation. However, these methods are typically laborious and time-consuming or require highly trained technicians to accurately read and interpret slides. For this purpose, a great deal of enthusiasm was raised by the application of the micronucleus test to uncultured exfoliated cells (Stich et al., 1992). Micronucleus arises from acentric fragments or whole chromosomes which are not included into the main nuclei of the daughter cells. The formation of micronuclei can be induced by substances that cause

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chromosome breakage (clastogens) as well as by agents that affect the spindle apparatus (aneugens) (Belien et al., 1995). Recently, we have applied this methodology with success in individuals exposed to dental X-ray (Ribeiro and Angelieri, 2008) or with malignant tumors undergoing chemotherapy (Minicucci et al., 2008). In the present study, we investigated the frequencies of micronucleated cells in oral mucosa from petrol station workers and control subjects using two different anatomical sites: cheek mucosa or the lateral border of the tongue. To monitor cytotoxic effects, pyknosis, karyolysis and karyorrhexis were also evaluated in this setting.

2. Material and methods

2.1. Subjects

The subjects of this study comprised a total of 23 healthy adults (22 men and one woman) with a mean age of 37.3 + 11.1 years working at petrol stations located in the urban area of Santos city, São Paulo state, Brazil. All participants work 40 h per week at least five years. The control group consisted of 23 healthy men volunteers with a mean age of 39.6 ± 13 years not exposed to petroleum derivatives. Individual characteristics of subjects were collected and included gender, age, habits and exposure to genotoxic agents.

Each person was interviewed concerning possible confounding factors and was excluded from this study when there was a lesion visible on the oral mucosa at the clinical examination, a history of cancer, previous radio- or chemotherapy, use of therapeutic drugs and exposure to diagnostic X-rays during the last 6 months.

The study was approved by the Ethics Committee of UNIFESP, Federal University of Sao Paulo. Informed consent was obtained from the individuals included in the study.

2.2. Micronucleus test in oral mucosa cells

Exfoliated oral mucosa cells were collected from buccal mucosa or lateral border of the tongue. After rinsing the mouth with tap water, cells were obtained by scraping the right/left cheek mucosa and right/left lateral border of the tongue with a moist wooden spatula. Cells were transferred to a tube containing saline solution (NaCl at 0.9% concentration in distilled water), centrifuged (800 rpm) during 5 min, fixed in 3:1 methanol/acetic acid, and dropped onto pre-cleaned slides. Later, the air-dried slides were stained using the Feulgen/Fast-Green method (Belien et al., 1995), and examined under a light microscope at 400× magnification. A total of one thousand cells were scored from each person from control and exposed groups as described elsewhere (Buajeeb et al., 2007).

2.3. Data analysis

Micronuclei were scored according to the criteria described by Sarto et al. (1987) as a parameter of DNA damage (mutagenicity). For cytotoxicity, the following nuclear alterations were considered: pyknosis, karyolysis and karyorrhexis. Results were expressed in percentage (%). Such analysis was established in a previous study conducted by our research group (Angelieri et al., 2007).

Table 1
Frequency of micronucleated cells (MNC) and other nuclear alterations (karyorrhexis, pyknosis and karyolysis) in buccal mucosa cells from petrol station attendants.

Groups	MNC (%)		Other nuclear alterations[a] (%)	
	No. of individuals	Mean ± S.D.	No. of individuals	Mean ± S.D.
Control group	23	0.04 ± 0.04	23	6.52 ± 3.84
Petrol station attendants	23	0.42 ± 0.06[b]	23	24.53 ± 6.87[b]

[a] Karyorrhexis, pyknosis and karyolysis.
[b] $p<0.05$ when compared to control group.

Table 2
Frequency of micronucleated cells (MNC) and other nuclear alterations (karyorrhexis, pyknosis and karyolysis) on the cells of lateral border of the tongue from petrol station attendants.

Groups	MNC (%)		Other nuclear alterations[a] (%)	
	No. of individuals	Mean ± S.D.	No. of individuals	Mean ± S.D.
Control group	23	0.04 ± 0.06	23	7.45 ± 3.81
Petrol station attendants	23	0.70 ± 0.09[b]	23	14.06 ± 5.87[b]

[a] Karyorrhexis, pyknosis and karyolysis.
[b] $p<0.05$ when compared to control group.

2.4. Statistical methods

The Mann–Whitney non-parametric test was used to compare the frequencies of micronuclei and other cellular alterations among the samples between exposed versus control groups using SigmaStat software, version 1.0 (Jadel Scientific, USA). The level of statistical significance was set at 5%.

3. Results

Tables 1 and 2 show the frequencies of micronucleated cells in petrol gas attendants and control individuals. In the control group, the mean frequency of micronucleated cells (MNC) was 0.04%. However, the group exposed to petroleum derivatives, the mean frequency of MNC was 0.42% in buccal mucosa cells and 0.70% in cells from lateral border of the tongue. Additionally, it was observed an increase of other nuclear alterations closely related to cytotoxicity as depicted by the frequency of karyorrhexis, pyknosis and karyolysis in both anatomic sites evaluated, i.e. buccal mucosa and lateral border of the tongue being the most pronunciated effect for the cheek mucosa.

In this study, the volunteers were considered smokers if they had smoked more than 5 cigarettes per day for at least 5 years. Herein, a total of six individuals were categorized as smokers in the experimental group and seven in the control group. Nevertheless, this study was not able to prove significantly the hypothesis of a relationship between increasing micronuclei count and smoking status between non-exposed subjects (control) and petrol station attendants either to buccal mucosa cells or to lateral border of the tongue, although smokers showed high values when compared to non-smokers. Also, cigarette smoke did not interfere with cytotoxicity induced by petroleum in both anatomic sites investigated. Such results are presented in Figs. 1 and 2. Overall, no interaction was observed between smoking and petroleum exposure.

Finally, exposure to known genotoxins was not related to any of the study participants. A total of 11 individuals use oral antiseptic solutions regularly. The daily

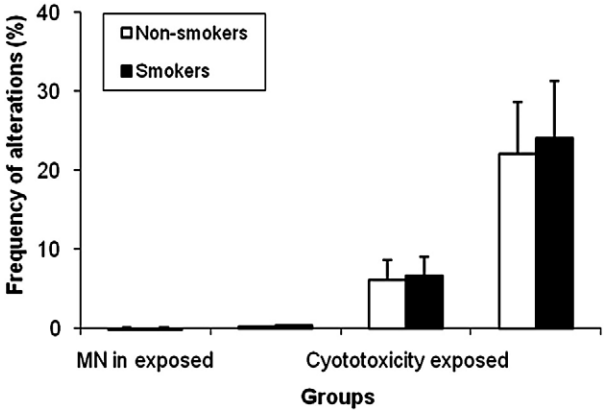

Fig. 1. Micronucleus and other nuclear alterations from buccal mucosa in ever-smokers and non-smokers from control and exposed groups. Results are expressed as Mean ± S.D. $p>0.05$.

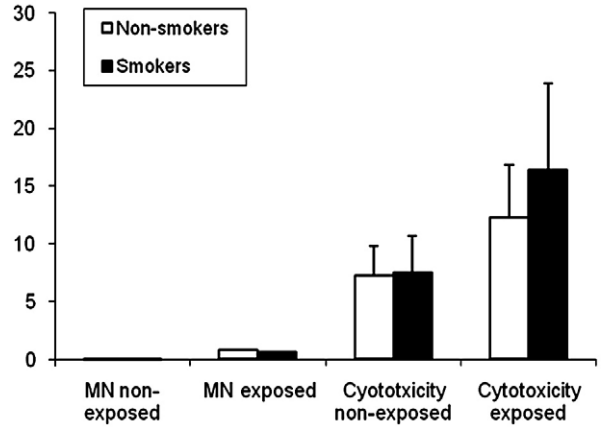

Fig. 2. Micronucleus and other nuclear alterations from lateral border of the tongue in ever-smokers and non-smokers from control and exposed groups. Results are expressed as Mean ± S.D. $p > 0.05$.

alcohol consumption was not considered in this study, because recall bias phenomenon has occurred.

4. Discussion

The aim of this study was to evaluate cytogenetic damage and cellular death induced by exposure to petroleum products. The investigation was conducted using the micronucleus test in oral exfoliated cells using two different oral anatomic sites. Micronucleus assay in exfoliated oral mucosa cells has been systematically used in genetic biomonitoring of populations exposed to several genotoxic chemicals, such as tobacco products, pesticides and alcohol consumption (Sarto et al., 1990; Bloching et al., 2000; Pastor et al., 2001; Nersesyan, 2006). The key advantage of the micronucleus assay is the relative ease of scoring, the limited costs and person–time required, and the precision obtained from scoring larger numbers of cells. To the best of our knowledge, the approach has not been demonstrated so far.

Micronucleated cell indexes may reflect genomic instability (Neri et al., 2003). The detection of an elevated frequency of micronuclei in a given population indicates increased risk of cancer (He et al., 2000). A number of experimental studies, as well as epidemiological evidence, indicate that gasoline and diesel engine exhausts are mutagenic and carcinogenic to laboratory animals and possibly to humans (Carere et al., 1995). It was expected that the micronucleus frequencies were significantly different between control and exposed subjects in this trial. Such findings are fully in line with other authors (Celik et al., 2003; Benites et al., 2006; Hallare et al., 2008). By comparison, an elevated level of cytogenetic damage in peripheral blood lymphocytes of workers with occupational exposure to petroleum and petroleum derivatives has been demonstrated using different genetic endpoints such as sister chromatid exchanges, DNA strand breaks and micronuclei (HoÈgstedt et al., 1991; Oesch et al., 1995; Bukvic et al., 1998). Conversely, some studies have demonstrated an absence for an effect between petrol attendants and controls (Carere et al., 1995; Pitarque et al., 1997). In a previous study performed by Surralles et al. (1997), a molecular cytogenetic analysis on buccal mucosa cells in benzene exposed workers showed no significant differences when compared to controls. Interestingly, our data revealed that the lateral border of the tongue was a more sensitive site to genotoxic insult when compared to buccal mucosa cells, since higher values were obtained in the tongue. These data are new in health sciences and, therefore, difficult to explain. By comparison, some studies have revealed that there are no differences in the micronucleus frequency from buccal mucosa or tongue cells in individuals exposed to dental X-ray or beedi smokers (da Silva et al., 2007; Suhas et al., 2004). Considering that >90% of all human cancers are of epithelial origin (Celik et al., 2003) and that the lateral border of the tongue is a high risk site for oral cancer (Kujan et al., 2006), we assumed that petroleum derivatives are able to induce genomic instability in oral cells, as a result of chromosomal breakage or loss, especially cells from the lateral border of the tongue.

To monitor cytotoxic effects, the frequencies of karyorrhexis, karyolysis and pyknosis were evaluated into this experimental design. Our results demonstrated that petroleum exposure was able to induce cellular death as depicted by statistically significant differences ($p<0.05$) between groups. Similar results were described by other researchers (Celik et al., 2003; Benites et al., 2006). Taken as a whole, such results support the notion that petroleum was also a cytotoxicant agent. It is important to stress that cytotoxicity interferes with micronucleus induction since some micronucleated cells are inevitably lost after cytotoxic injury confirming, therefore, our present findings, because higher cytotoxic levels in buccal mucosa cells were detected when compared with lateral border of the tongue. Following the same rationale, it has been postulated that repeated exposure to cytotoxicants can result in chronic cell injury, compensatory cell proliferation, hyperplasia and ultimately tumor development (Swenberg 1993). In fact, a correlation between cell proliferation and induction of cancer is assumed (Sugano et al., 2001). Probably, proliferation may increase the risk of mutations within target cells, and is also important in selective clonal expansion of (exogenously or endogenously) initiated cells from preneoplastic foci to as far as malignant tumors (Mally and Jagetia, 2002).

Tobacco is known to contain various genotoxic chemical and smoking is a well-documented cause of cancer including the oral cavity (Speit et al., 2003). For this reason, smokers and non-smokers are comparatively investigated because smoking is supposed to be a relevant exposure to genotoxins (Topinka et al., 1989). Our results demonstrated no effect in micronuclei frequency between smokers and non-smokers, either to exposed or to control groups. Therefore, no interaction was observed between smoking and petroleum exposure. By comparison, an earlier study conducted by our group has revealed no differences in the micronucleus frequency between smokers and non-smokers submitted to dental X-ray (Ribeiro and Angelieri, 2008). In fact, several works have failed to show any positive carcinogenic effect of smoke. One study has even reported no differences in the induction of micronuclei between smokers and non-smokers, while others have shown that smokers had less DNA damage than non-smokers (Sram et al., 1988). On the other hand, some authors have postulated increased DNA damage in heavy smokers (Schwartz et al., 2003). In addition, exposure to nicotine caused a statistically significant increase of micronucleus frequency in human gingival fibroblasts *in vitro* (Argentin and Chichetti, 2004). It is important to keep in mind that *in vitro* studies do not consider the complex *in vivo* situation. In this regard, such findings should be interpreted cautiously.

Besides the power of the statistical analysis as a critical factor for the determination of an effect, various additional explanations (including seasonal and regional differences) for the reported discrepancies have been proposed (Jagetia et al., 2001). Particularly, some confounding factors are important to be considered to human cytogenetic studies. Viruses, alterations in the immune system, failures in DNA repair system and inter-individual variations have already been associated with increased frequencies of chromosome aberrations (Xu et al., 1999). Furthermore, an age-related increase of micronuclei has been postulated (Torres-Bugárin et al., 2003). Due to the homogeneity in casuistics, it was not possible to correlate the frequency of micronucleated cells with the age in this setting.

On the basis of the current understanding that smoking exerts a negative influence on the oral mucosa, it was hypothesized that oral cells would also be killed by smoking. The main reason for such an assumption is that it has been argued that some tobacco products affect apoptosis process induced by various stimuli including ultraviolet light (Sugano et al., 2001) and chemotherapeutic agents in cancer cell lines (Onoda et al., 2001). Moreover, some authors have argued that nicotine

is able to prevent apopotosis in human gingival fibroblasts *in vitro* (Argentin and Chichetti, 2004) and *in vivo* using short-term assay in rodents (Assis et al., 2005). Our results showed that smokers did not present distinct values for cytotoxicity parameters when compared to non-smokers. Conversely, Schwartz et al. (2003) have suggested an increase of apoptosis in heavy smokers. Possible explanations for the diverging results may be found in differences concerning the methodology and/or population characteristics as well as the size of casuistics. This issue requires further investigation.

In conclusion, the results of the present study suggest that gas petrol attendants comprise high risk group since petroleum derivatives can induce mutagenic and cytotoxic effects in oral mucosa cells. It seems that the lateral border of the tongue is a more sensitive site to geno- and cytotoxic insult induced by petroleum derivates. Since DNA damage and cellular death are considered to be prime mechanisms during chemical carcinogenesis, these data may be relevant to risk assessment in protecting human health and preventing carcinogenesis. However, further elucidation in forthcoming studies is welcomed.

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