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ORIGINAL ARTICLE

Biomonitoring of oral epithelial cells in smokers and non-smokers submitted to panoramic X-ray: comparison between buccal mucosa and lateral border of the tongue

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Abstract 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 smokers and non-smokers submitted to dental X-ray using two anatomic sites: buccal mucosa and lateral border of the tongue. A total of 15 heavy smokers and 17 non-smokers were submitted to panoramic dental radiography for orthodontic reasons. 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 no significant statistically differences ($p > 0.05$) of micronucleated oral mucosa cells before versus after X-ray exposure for both oral sites evaluated either to smokers or to non-smokers. X-ray exposure was able to increase other nuclear alterations closely related to cytotoxicity such as karrhyorexis, pyknosis, and karyolysis for two groups evaluated. Nevertheless, the most pronunciated effects were found to lateral border of the tongue of smokers. In summary, these data indicate that panoramic X-ray is able to induce cellular death in oral mucosa cells. It seems that lateral border of the tongue is more sensitive site to cytotoxic insult induced by ionizing radiation combined with continuous cigarette smoke exposure.

Keywords Buccal mucosa cells · Tongue cells · Cigarette smoke · X-ray · Micronucleus test

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Introduction

Panoramic dental radiography is a specialized technique used to produce a flat representation of the curved surfaces of the jaws. It is an excellent and widely performed technique for providing an overview of the dentition, generalized pathology such as periodontitis, odontogenic, and non-odontogenic lesions of the jaws [1].

Accumulating evidence suggests that cigarette smoke is a complex mixture of 3,800 compounds including high concentrations of both free radicals and chemical compounds that readily react to form other reactive substances, some of them well-known genotoxic agents [2]. DNA mutations are found in tumors typically associated with smoking, such as those located in oral cavity, oropharyngeal, and lung [3]. Particularly, studies of epithelial tissues from smokers have shown elevated measures of DNA damage and increased DNA mutations when compared with epithelial tissue from non-smokers [4].

Biomarkers have been used in medicine and toxicology for many years to assist in diagnosing, staging disease, as well as to evaluate 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 [5]. 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 [6, 7]. 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 chromosome breakage (clastogens) as well as by agents that affect the spindle apparatus (aneugens) [8]. Recently, we have demonstrated that dental X-ray is able to induce cytotoxicity in buccal cells either to non-smokers or to children [9]. However, it would be interesting to know if, and to what extent, heavy smokers compose a more susceptible group following X-ray exposure in distinct sites of oral cavity, particularly because there are no previous reports.

As a result, and because of limited evidence, the aim of this study was to evaluate the frequencies of micronucleated cells in oral mucosa cells of heavy smokers submitted to panoramic dental radiographies using two oral anatomic sites: cheek mucosa and lateral border of the tongue. To monitor cytotoxic effects, pyknosis, karyolysis, and karyorrhexis were also evaluated in this setting. Certainly, such results will contribute to a better understanding of effects induced by X-ray upon cellular system in individuals continually exposed to known genotoxic agents.

Material and methods

Subjects

The subjects of this study comprised a total of 15 healthy adults (nine men and six women) with a mean age of 37.7± 6.5. All individuals were heavy smokers (consumption of 20 or over 20 cigarettes/day) for at least 10 years. Furthermore, seventeen adults (11 men and six women) with a mean age of 39.6±5.4 were included as non-smokers. All patients were submitted to panoramic dental radiography at the Orthodontics Department of the Sao Paulo Metodista University, UMESP, SP, Brazil. All panoramic dental radiographies were requested by the dentist and were performed with Siemens Orthophos equipment (Erlangen, Germany), system 250-71 kV/ 15 mA/14 s/110mGycm^2. The entrance dose was 0.08R. The study was approved by the Human Ethics Committee of UMESP, Sao Paulo Metodista University. Informed consent was obtained from the individuals included in the study.

Micronucleus test in oral mucosa cells

Exfoliated oral mucosa cells were collected immediately before the X-ray exposure and after 10 days. After rinsing the mouth with tap water, cells were obtained by scraping the right/left cheek mucosa or left/right lateral border of the tongue with a moist wooden spatula. Cells were transferred to a tube containing saline solution, 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, and examined under a light microscope at ×1,000 magnification to determine the frequency of micronucleated cells. Two thousand cells were scored from each patient for each sampling time (before and after X-ray exposure).

Data analysis

Micronuclei were scored according to the criteria described by Sarto et al. [10] 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 [11].

Statistical methods

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

Results

Figure 1 show the frequencies of micronucleated cells in non-smokers and smokers following X-ray exposure. Before X-ray exposure (control), the mean frequency of micronucleated cells was 0.05%. No significant statistical

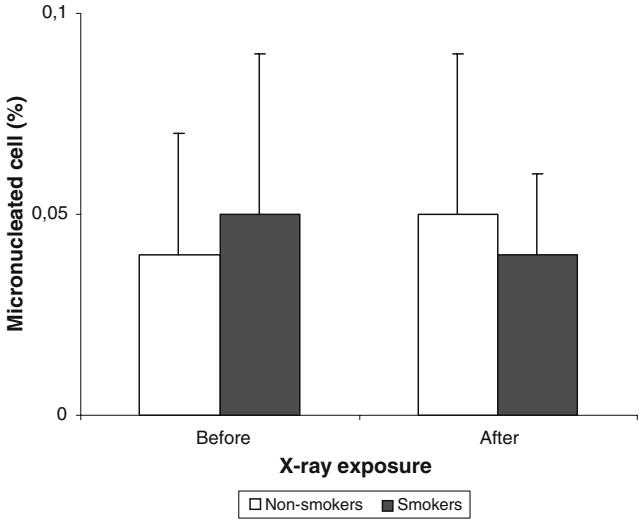

Fig. 1 Micronuclei frequencies from cheek mucosa of smokers and non-smokers exposed to X-ray, $p>0.05$

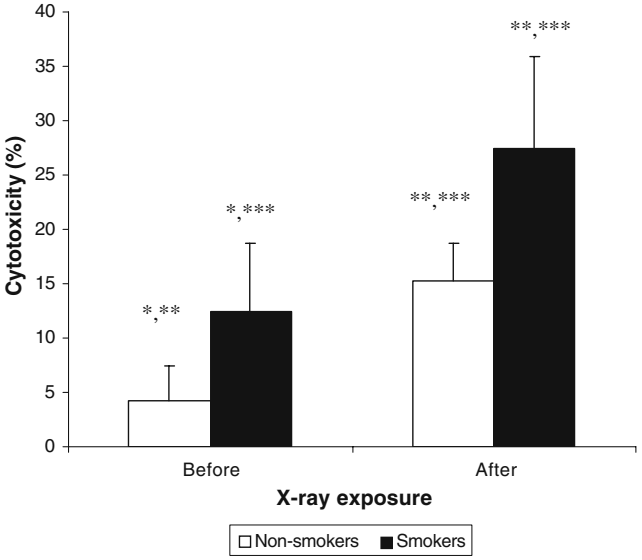

Fig. 3 Cytotoxicity parameters from cheek mucosa of smokers and non-smokers exposed to X-ray. *, **, ***, $p<0.05$

differences ($p>0.05$) were noticed to micronucleated cells after X-ray exposure, independent of oral site evaluated, i.e., cheek mucosa or lateral border of the tongue for both groups evaluated. Such data are displayed in Fig. 2.

However, X-ray exposure was able to increase other nuclear alterations closely related to cytotoxicity such as karrhyorexis, pyknosis, and karyolysis of non-smokers and heavy smokers. Such differences were detected in both anatomic sites evaluated (buccal mucosa and lateral border of the tongue). Such data are summarized in Figs. 3 and 4. Figure 5 shows micronucleated cell. Figure 6 displays karyorrhexis, pyknosis, and karyolysis.

Finally, exposure to known genotoxins was not related to any of the study participants. A total of eight individuals use oral antiseptic solutions regularly. The daily alcohol consumption was not considered in this study, because recall bias phenomenon has occurred.

Discussion

The aim of this study was to comparatively evaluate cytogenetic damage and cellular death induced by exposure to X-ray in smokers and non-smokers. The investigation was conducted using the micronucleus test in oral exfoliated cells using two different oral anatomic sites. To the best of our knowledge, the approach has not been addressed so far.

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

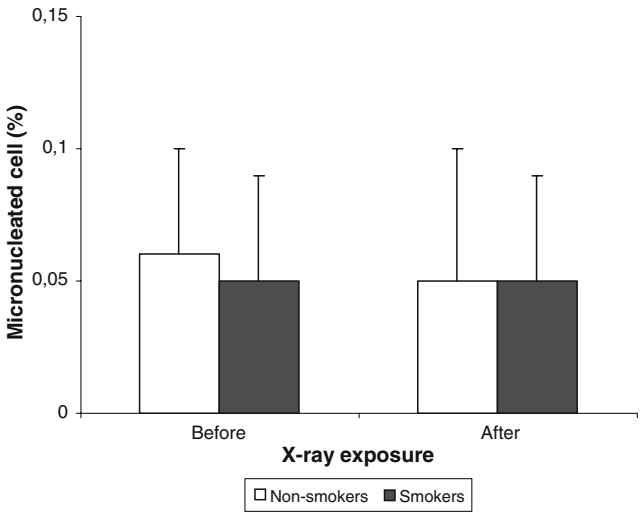

Fig. 2 Micronuclei frequencies from lateral border of the tongue of smokers and non-smokers exposed to X-ray, $p>0.05$

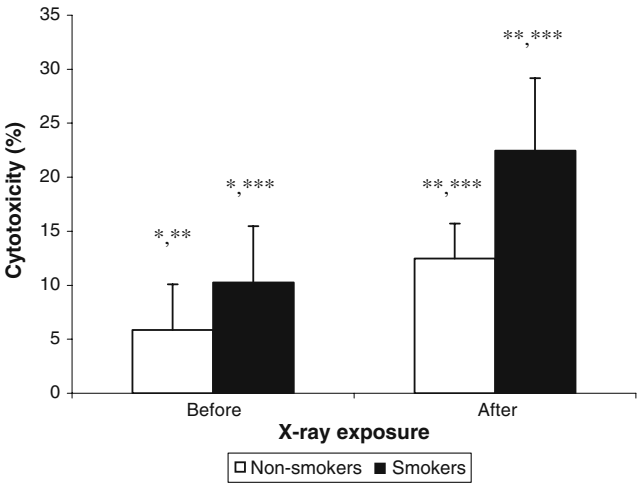

Fig. 4 Cytotoxicity parameters from lateral border of the tongue of smokers and non-smokers exposed to X-ray. *, **, ***, $p<0.05$

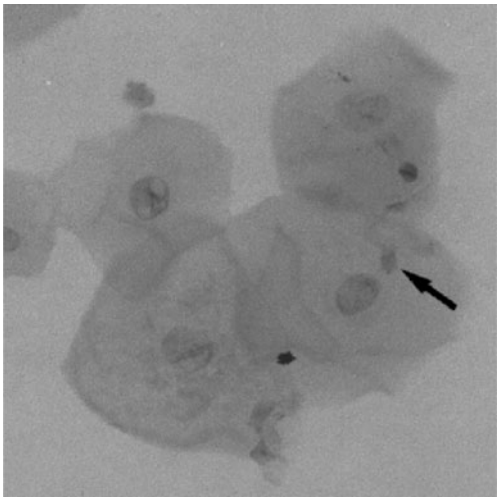

Fig. 5 Micronucleated cell (*arrow*); ×100 magnification, Feulgen/Fast green stain

numbers of cells. The measurement of the frequency of micronuclei induced in cells by mutagen agents is widely used for analysis of cytogenetic damage [8]. Micronuclei contain genetic material that is lost from the genome during mitosis, as a result of a clastogen or aneugen occurrence [8]. Hence, there will arise bigger micronuclei from whole chromosomes as a follow-up to damaging of the spindle apparatus of the cell (aneugen). Smaller micronuclei are the result of structural aberrations and consist of chromosomal fragments [12]. Damages that lead to the formation of micronuclei takes place in the basal layer of the epithelial tissue, where cells undergo mitosis. Rapid turnover of epithelial tissues brings the cells to the surface whey they exfoliate. As a result, the maximal rate of micronuclei formation in exfoliated cells is seen 1–3 weeks after exposure to the genotoxic agent [13]. For this reason, evaluation was done 10 days after X-ray exposure in this study.

Genomic damage is probably the most important fundamental cause of developmental and degenerative diseases. It has been well-established that genomic damage is produced by environmental exposure to genotoxins, medical procedures (e.g., radiation and chemicals), micronutrient deficiency (e.g., folate), lifestyle factors (e.g., alcohol, smoking, drugs, and stress), and genetic factors such as inherited defects in DNA metabolism and/or repair [14]. Micronucleated cell indexes may reflect genomic instability [15]. The detection of an elevated frequency of micronuclei in a given population indicates increased risk of cancer [16]. However, cell types that repair DNA damage efficiently are likely to show lower levels of residual damage than cells less proficient in DNA repair [17]. Buccal cells have been shown to have limited DNA repair capacity relative to peripheral blood lymphocytes, and therefore may more accurately reflect genomic instability events in epithelial tissues [18].

Tobacco is known to contain various genotoxic chemical and smoking is a well-documented cause of cancer including the oral cavity [19]. In our recent paper [20], we were able to evaluate if panoramic dental X-ray is able to induce mutagenicity or cytotoxicity by means of micronucleus test. The casuistics comprised a total of 39 individuals being nine heavy smokers only. Unfortunately, the total number of smokers in this trial was insufficient for a positive response since our hypothesis was that tobacco smoke could interfere with micronucleus frequency or cytotoxicity parameters following X-ray exposure. For this reason, we were able to design a new study containing 15 heavy smokers. Now, we were able to demonstrate that cigarette smoke induced cellular death as depicted by statistically significant differences ($p \leq 0.05$) between smokers and non-smokers (Figs. 3 and 4). Such results were described by other researchers [21]. It has been hypothesized that oral cells are died by smoking. The main reason

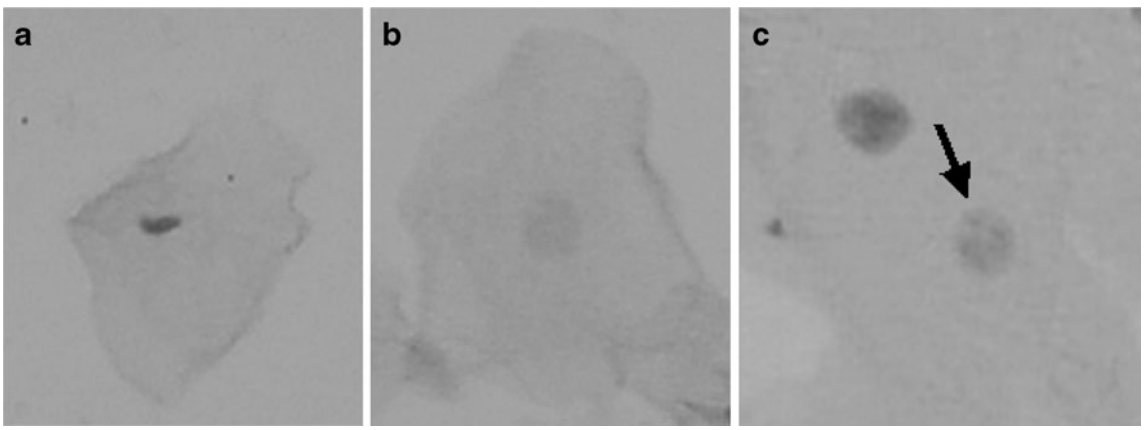

Fig. 6 Cytotoxicity parameters evaluated in this study: **a** pyknosis, **b** karrhyorexis, and **c** karyolysis; ×100 magnification, Feulgen/Fast green stain

for such an assumption is that it has been argued that some tobacco products affect apoptosis process induced by various stimuli including ultraviolet light [22] and chemotherapeutic agents in cancer cell lines [23]. Moreover, some authors have argued that nicotine is able to prevent apoptosis in human gingival fibroblasts in vitro [2]. Conversely, Schwartz et al. [24] has suggested an increasing of apoptosis in heavy smokers. Possible explanations for the diverging results may be found in differences concerning to methodology and/or population characteristics as well as the size of casuistic. This issue requires further investigation.

Our results demonstrated no increase in micronucleus frequency between non-smokers and smokers. In fact, several works have failed to show any positive mutagenic effect of smoke. Some studies have reported no differences in the induction of micronuclei between smokers and non-smokers [21], while others have shown that smokers had less DNA damage than non-smokers [6]. On the other hand, some authors have postulated increased DNA damage in heavy smokers [24]. In addition, exposure to nicotine caused a statistically significant increase of micronucleus frequency in human gingival fibroblasts in vitro [25]. 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.

It was surprising that the micronucleus frequencies were not significantly different before and after X-ray exposure for all groups evaluated in this trial (Figs. 1 and 2). By comparison, previous study conducted by our research group has demonstrated no increase of micronuclei in children or in adults exposed to panoramic X-ray [20]. Such findings are fully in line with other authors [26, 27]. Conversely, some authors have reported higher rates of cytogenetic damage induced by X-ray [16]. Biomonitoring studies of populations exposed to X-ray are quite difficult and rather specific because each population is exposed to different doses of radiation. This could explain why some studies find an increase of genetic damage in populations exposed to X-ray. Based on our results found, we postulated the lack of clastogenic and/or aneugenic effects related to the dental panoramic radiography in healthy individuals continuously exposed to tobacco products or not.

Moreover, our results demonstrated that panoramic dental radiography was able to induce cellular death as depicted by statistically significant differences ($p<0.05$) between values before versus after X-ray exposure for both groups evaluated being the most pronunciated effect for lateral border of the tongue. Considering that >90% of all human cancers are of epithelial origin and that lateral border of the tongue is a high-risk site for oral cancer [28], we assumed that X-ray is able to induce cellular death in oral cells especially cells from lateral border of the tongue. It is important to stress that cytotoxicity interferes with micronucleus induction since some micronucleated cells are inevitably lost after cytotoxic insult, confirming, therefore, to the lack of mutagenic effect induced by X-ray. Nevertheless, it has been postulated that repeated exposure to cytotoxicants can result in chronic cell injury, compensatory cell proliferation, hyperplasia, and ultimately tumor development [29]. In fact, a correlation between cell proliferation and induction of cancer is assumed [17]. Probably, proliferation may increase the risk of mutations within target cells, and also be important in selective clonal expansion of (exogenously or endogenously) initiated cells from preneoplastic foci, and eventually tumors [17]. Taken together, it seems that smoking is able to induce strong cytotoxicity in oral mucosa cells, especially cells from lateral border of the tongue in individuals exposed to X-ray.

Besides the power of the statistical analysis as a critical factor for the determination of putative outcome, various additional explanations (including seasonal and regional differences) for the reported discrepancies have been proposed [17]. 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 [30]. Furthermore, an age-related increase of micronuclei has been postulated [31]. Due to the homogeneity in casuistics, it was not possible to correlate the frequency of micronucleated cells with the age in this setting.

In conclusion, the results of the present study suggest that panoramic X-ray is able to induce cellular death in oral mucosa cells. Smokers comprise high-risk group since high cytotoxicity levels were found, especially in tongue mucosa cells. Since DNA damage and cellular death are considered to be prime mechanisms during chemical carcinogenesis, these data may be relevant in risk assessment for protecting human health and preventing carcinogenesis. However, further elucidation in forthcoming studies is welcomed.

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Conflict of interest The authors declare that they have no conflict of interest.

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