Original Article

Evaluation of Genotoxicity in Patients Subjected to Panoramic Radiography by Micronucleus Assay on Epithelial Cells of the Oral Mucosa

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Abstract

Objective: Radiography is one of the most valuable diagnostic tools used in comprehensive dental care. Although there is no safe level of radiation exposure, the possible risk associated with exposure to radiation, must be elucidated. To date, a variety of assays have been proposed to assess the mutagenic potential of genotoxicants; however, these methods are typically laborious and time consuming. The aim of the present study was to evaluate the possible genotoxic effect of routinely used panoramic radiation exposure in exfoliated epithelial cells as measured by the formation of micronuclei and to compare the genotoxicity of X-rays on keratinized epithelial gingival cells and the nonkeratinized buccal epithelial cells.

Materials and Methods The study included 53 healthy individuals with a mean age of 25.21 ±12.67 years. Specimens of exfoliated epithelial cells were collected from patients subjected to panoramic radiography before and 10 days after radiation exposure. The cells were stained with Giemsa and evaluated for micronuclei by scoring 1000 cells per slide.

Results: In our study, the genotoxic effect of radiation exposure from panoramic radiography showed a statistically significant increase in the MN frequency in buccal epithelial cells. A significant correlation was observed between the age of the subjects and micronuclei, although no such correlation was found between gender and micronuclei count.

Conclusion: MN test serves as a simple biomarker indicating the direct exposure to DNA damaging agents such as ionizing radiation, emphasizing great sensitivity even for exposure to low doses during radiation screening. Thus, panoramic dental radiography should be cautiously used only when necessary.

Key Words: Panoramic Radiography; Micronucleus Tests; Epithelial Cells; Ionizing Radiation

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INTRODUCTION

Carcinogenesis is a multistep process governed by genetic or epigenetic mechanisms and signalling pathways that leads to the change in morphology and cellular behaviour resulting in mutations related to the control of cell division, cell death, and metastatic potential [1]. Emerging information suggests that

exposure to genetic and environmental agents can increase the risk of cancer. When the normal function of DNA repair genes is altered, as a result of mutations, the risk of malignant transformation increases [2]. Among these agents, ionizing radiation forms the bulk of contribution to human exposure because of its wide use for diagnostic and therapeutic purposes [3]. Ionizing radiation is a potent mutagenic agent, inducing both gene mutations and chromosomal aberrations [2]. X-rays have been widely used for diagnosis in medical and dental practice. Therapeutic radiology has its own severe effects and an entity called stochastic effects also exists that can cause malignancy. Dentists now a days use panoramic radiographs as a screening tool for diagnosing dental arch and tooth diseases and during panoramic radiography, gingival and buccal epithelial cells serve as an alternative source to peripheral lymphocytes for evaluating genotoxic and cytotoxic effects, as they are under direct radiation exposure at dental radiographic examination [4]. In addition, unlike peripheral lymphocytes, epithelial cells can be easily and rapidly sampled, do not have to be cultivated, do not require stimulation or metaphase preparations; therefore, application of the micronucleus test in epithelial cells is considered as a sensitive tool to biomonitor genetic damage in populations exposed to several genotoxic agents [1]. To date, a variety of assays have been proposed as potential biomarkers in biomonitoring studies for evaluating the mutagenic potential of physical, chemical and biologic agents.

But these assays are not cost effective, are time-consuming and require skilled technicians to precisely interpret the slides. For this reason, a validated, sensitive, non-invasive and economical technique was necessary. Thus, a great deal of enthusiasm was raised by application of the micronucleus test to assess DNA damage induced by low-level ionizing radiation [6].

Nowadays, a lot of work is being done on micronucleus (MN) assay, especially in the field of oral cancer [7]. MN takes its origin from chromosome fragments or whole chromosomes that lag behind at anaphase during nuclear division [8]. Micronuclei can be detected in exfoliated oral epithelial cells and they reflect the ongoing process of DNA damage [9]. They may arise from the whole lagging chromosome or the acentric chromosome fragment. They do not integrate in the daughter nuclei during mitosis and appear in the cytoplasm as additional small nuclei. Elucidating the genotoxic effects induced by X- rays is relevant to identifying the degree of cancer risk and minimizing the potential risks to patients and clinicians. Thus, in order to contribute to the effects of routinely exposed X-rays upon the cellular system; in the present study the frequencies of micronucleated cells have been evaluated in the buccal and gingival epithelial cells of patients who had undergone panoramic dental radiography.

MATERIALS AND METHODS

In the present study patients visiting the Department of Oral Medicine and Radiology Rama Dental College were evaluated clinically using a mouth mirror and a probe. Fifty-three patients who were advised for OPG were considered as the study group. Patients with any potentially malignant or malignant lesions, patients who had undergone diagnostic radiographs in the previous 6 months, radiotherapy or chemotherapy, patients who had regular use of antiseptics and antioxidants, a history of tobacco and alcohol consumption, and genetic disorders were all excluded from the study. Cells from buccal mucosa and keratinized gingiva were obtained from 53 patients subjected to panoramic radiography. They answered a questionnaire before radiographic examination. Demographic data of the patients was collected and informed consent was obtained from all subjects after explaining about

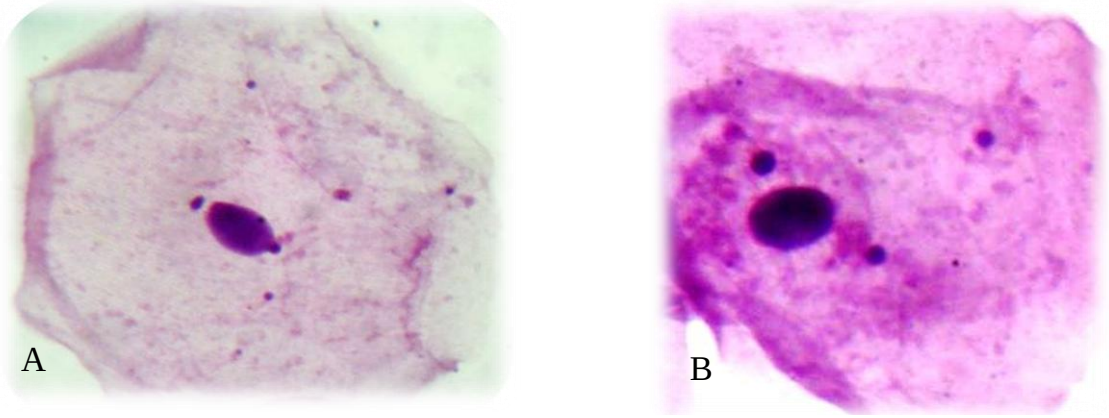

Fig1. Photomicrograph showing Giemsa-stained mucosal cells with cytoplasmic micronuclei; a, x 100 magnification, b, x 400 magnification

the study. The panoramic dental radiographs were performed with gendex orthoralix system, 74 kV and 10 mA, 12s with output dose rate of 0.325 m Gy/s at 70 kV, 10 mA. The university's ethical clearance committee approved the study.

Cell Sampling and Slide Preparation

Each of the 53 subjects sat comfortably on the dental chair and buccal cells from the inside of both cheeks and gingival cells from the keratinized mucosa of the upper dental arch were collected immediately before x-ray exposure and 10 days after exposure.

After rinsing the mouth with water, cells were obtained by gentle scraping with a metal spatula and transferred to a tube containing 25 ml buffer solution (0.1M EDTA, 0.01M Tris HCl, 0.02M NaCl) pH 7.0. Cytological smears were prepared by washing the cells thrice in the buffer solution by centrifugation at 800rpm for 5 minutes.

This washing procedure helps to remove bacteria and cell debris. Cell suspension was dropped on to clean slides and cell density was checked using a light microscope. The pellet was dried, fixed in 80% cold methanol and smears were stained with 10% Giemsa solution. The slides were prepared for each subject and covered with cover glass using mountant.

Micronucleus Analysis

All slides were observed by a senior pathologist in a pathological laboratory under light microscope using low magnification for screening (X100) and higher magnification for counting of micronuclei (X400). The frequency of micronuclei in the epithelial cells was evaluated by scoring 1000 cells with intact nuclei, full cytoplasm and cell boundaries on each slide. The criteria for designating micronuclei was scored according to Tolbert et al. [10] which includes:

(a) Rounded smooth perimeter suggestive of a membrane;
(b) Less than one third of the diameter of the associated nucleus, but large enough to discern shape and colour;
(c) Staining intensity similar to that of the nucleus;
(d) Texture similar to that of the nucleus;
(e) Same focal plane as the nucleus; and
(f) Absence of overlap with, or bridge to, the nucleus.

Only those structures fulfilling the above mentioned- criteria were recorded as micronuclei. Micronuclei were seen as individualized bodies that were separated from the main nucleus within the cytoplasm (Figure 1). The data collected have been statistically analyzed and tabulated using SPSS 16.

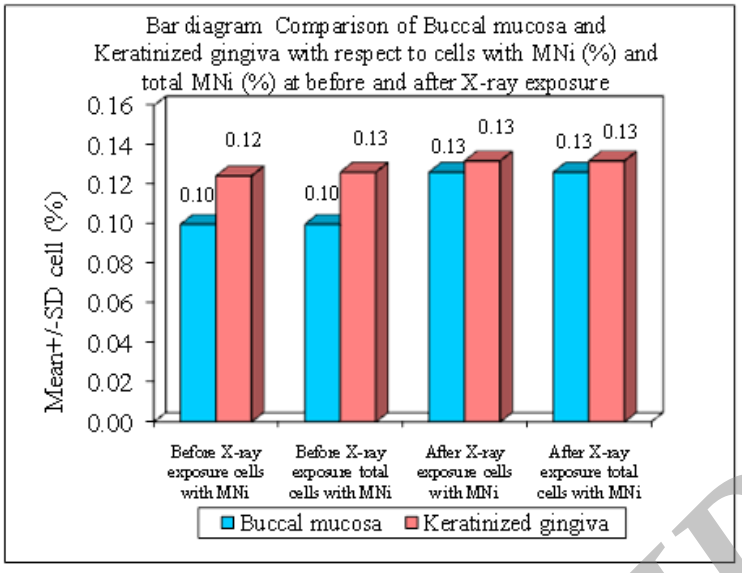

Fig 2. Bar diagram showing comparision of cells with Mni and total Mni in buccal mucosa and keratinized gingva before and after exposure

The tests used were t tests, Mann Whitney U test and Wilcoxon test and the values were represented in number (%) and mean and SD. Wilcoxon sign rank test was used to find out the significance of differences between the number of cells with Mni and total number of Mni before and after exposure in the buccal mucosa and keratinized gingiva, respectively. To determine the association of age with micronucleus count, before and after exposure in the buccal mucosa and the keratinized gingiva, groups 1 and 2 were compared using Mann-Whitney U test. The student's unpaired t test was used to assess the significance of difference between genders with respect to the frequency of micronuclei. Similarly, the comparison between buccal mucosa and keratinized gingiva with respect to cells with micronuclei and total micronuclei before and after exposure was done by unpaired t test. P-value of 0.05 or less was considered for statistical significance.

RESULTS

The sample included 53 subjects (21 males and 32 females). The mean age of the subjects was 25.21 ±12.67 years.

The association of the frequency of micronuclei with the gender of investigated subjects was tested by unpaired t test.

No association between the frequency of micronuclei and gender was found after exposure in the buccal mucosa and keratinized gingiva respectively. In our study comparison of age groups (<25 years and ≥25 years) with respect to cells with MNi (%) and total MNi (%) before and after X-ray exposure in the buccal mucosa and keratinized gingiva was done by Mann-Whitney U test. Significant difference was obtained before exposure (p = 0.0277) and after exposure (p=0.0203) when cells with micronuclei and the number of total Mni before (p=0.0209) and after exposure (0.0203) were compared in different age groups in the keratinized gingiva (Table 1), whereas no association between the frequency of total micronuclei and age was found in the buccal mucosa (p=.5020). In the present study, Table 2 shows the mean percentage of the number of cells with Mni and the total number of micronuclei before and after exposure in the buccal mucosa were 0.1000 ± 0.0899 and 0.1264 ± 0.0812, respectively with a mean difference of -0.0264 ±0.0524.

Table 1. Comparison of Age Groups (<25 Years and ≥25 Years) with Respect to Cells with MNi (%) and Total MNi (%) Before and After X-Ray Exposure in Keratinized Gingiva by Mann-Whitney U Test

X-Ray Exposure	Variable	Age Group	Mean (%)	SD (%)	U-Value	Z-Value	P-Value
Before	Cells with MNi	<25 years	1.4211	0.8584	173.50	-2.2016	0.0277*
		≥25 years	0.8000	0.5606			
	Total Mni	<25 years	1.4474	0.8605	168.00	-2.3102	0.0209*
		≥25 years	0.8000	0.5606			
After	Cells with MNi	<25 years	1.5000	0.8302	167.50	-2.3200	0.0203*
		≥25 years	0.8667	0.5164			
	Total Mni	<25 years	1.5000	0.8302	167.50	-2.3200	0.0203*
		≥25 years	0.8667	0.5164			

Table 2. Comparison of Before and After X-Ray Exposure in the Buccal Mucosa with Respect to Cells with MNi (%) and Total MNi (%) by Wilcoxon Matched Pairs Test

	X -Ray Exposure	Mean (%)	SD (%)	Mean Diff.	SD Diff.	Z-Value	P-Value
Cells with Mni	Before	1.0000	0.8987				
	After	1.2642	0.8122	-0.2642	0.5244	2.8961	0.0038*
Total Mni	Before	1.0000	0.8987				
	After	1.2642	0.8122	-0.2642	0.5244	2.8961	0.0038*

Table 3. Comparison of Before and After X-Ray Exposure in Keratinized Gingiva with Respect to Cells with MNi (%) and Total MNi (%) by Wilcoxon Matched Pairs Test by Ranks

	X-Ray Exposure	Mean (%)	SD (%)	Mean Diff.	SD Diff.	Z-Value	P-Value
Cells with Mni	Before	1.2453	0.8299				
	After	1.3208	0.8032	-0.0755	0.3310	1.46759	0.1422
Total Mni	Before	1.2642	0.8355				
	After	1.3208	0.8032	-0.0566	0.3048	1.2136	0.2249

The p value is <0.05 thus indicating that there was a statistically significant increase (p = 0.0038) in the total number of MNi after exposure in the buccal mucosa. No statistically significant increase (p = 0.2249) was observed in the total number of MNi after exposure in the keratinized gingiva (Table 3). In our study, while comparing total Mni percentage in the buccal mucosa and the keratinized gingiva by unpaired t test, there was no statistically significant increase (p= 0.1201) before and after exposure (p= 0.7190) (Figure 2).

DISCUSSION

MNi are an early indicator of carcinogenesis. Ionizing radiation is a well known mutagen and carcinogen in the human population. In spite of the mutagenic potential, this kind of radiation is an important tool for diagnosing diseases and is used in medical and dental practice [11].

Panoramic radiography is the most commonly advised diagnostic aid. In the present study, making use of exfoliated epithelial cells for assaying MN can be arguably explained on two bases. First, since epithelial cells form the basis of approximately 90% of human cancers, they are the preferred target sites for early genotoxic events induced by carcinogenic agents that gain their entry into the body by way of inhalation and ingestion. Secondly, epithelial cells can be easily collected from the oral cavity and have a potential to evaluate genotoxic damage in humans [7]. Therefore, application of the micronucleus test in epithelial cells is considered as a sensitive tool for biomonitoring genetic damage in the human population. The samples with exfoliated cells were collected 10-12 days after radiation exposure, which is the period required for their replacement with basal cells from the squamous epithelium [12,13].

Heddle (1973) initially described the well-established criteria for identifying MN but cell inclusion criteria was not provided by heddle. Stich and Rosin (1983) established the criteria for inclu-sion of cells for MN frequency, after which Tolbert et al. developed the criteria for scoring the cells and it is the most widely used criteria for evaluation of MN frequency till today [3,14,15] and this criteria was used in the present study.

Several staining methods have been used for the evaluation of micronucleus. Although DNA-specific stains are preferred for staining MN, the most commonly used staining procedure for identifying DNA of the nucleus and MN is the feulgen stain. Feulgen stain is favored by many investigators because of its DNA specificity. However, the staining procedure is a relatively lengthy procedure, it is also technique sensitive and may lead to under scoring of MN [16, 17]. Therefore, in the present study, we used Giemsa stain for assessing the micronuclei. In the present study, the MN occurrence with age was evaluated in the buccal mucosa and the keratinized gingiva and there was no significant association between age and the cells with Mni (p=0.2284) and total Mni (p=0.5020) before and after exposure in the buccal mucosa, whereas there was a statistically significant association of micronuclei frequency with age in keratinized gingiva before (p=0.0209) and after exposure (p=0.0203). Popova et al. (2007) [4] found a significant relationship between age and micronucleus frequency that is consistent with our study. It has been postulated that the age effect could reflect a progressive increase in spontaneous chromosome instability, associated with the accumulation of DNA damage due to an age-related decline in DNA repair capacity. The effect could also be explained by the fact that during aging, there is an increase in chromosome loss [18]. Conflicting results were reported towards the association between age and MN occurrences [2, 4].

Interestingly, although the percentage of females (70%) in each decade we studied was more compared to males, we did not find any

statistically significant difference between males and females for MNC's and Mni. The MN frequency in both males and females was almost equal. Thus, we did not find any association between gender and the occurrence of MN that is in accordance with the studies conducted by Popova et al. (2007) [4] and Cerquira et al. (2008) [2].

In the present study, a statistically significant increase in the frequency of cells with MNi and total MNi after exposure to OPG was observed from 0.10% to 0.13% ($p = 0.0038$) in the buccal mucosa. Similar studies were conducted by Fernanda Angelieri (2007) [6] and Popova et al. [4] in the buccal mucosa that evaluated the MN frequencies before exposure as 0.04±0.06% and 2.34±1.49%, respectively and after exposure as 0.05±0.06 and 2.81±1.64%, respectively. Although these studies showed an increase in MN frequency after panoramic radiographic exposure, the difference noted was not statistically significant, whereas in a study performed by Waingade et al. (2012) [16] on buccal epithelial cells, a statistically significant difference in MN frequency before and after exposure was reported, which was comparable to the present study. A higher MN frequency observed in our study could be explained by the fact that the buccal epithelial cells are under direct radiation exposure making it a primary target to radiation-induced damage as proposed by Popova et al. (2007) [4]. In our study, there was no statistically significant increase ($p = 0.2249$) in the number of cells with Mni and total number of MNi after exposure in keratinized gingiva. Cerqueira et al. (2008) in a study on gingival epithelial cells reported a statistically significant difference in MN frequency before and after exposure, whereas in a recent study conducted by Pai et al. (2012) [19] on gingival cells, there was an increase in MN frequency that was not statistically significant. Similarly, Da Silva et al. [20] have demonstrated that panoramic radiography increases the number of nuclear anomalies (except micronuclei),with statistically significant differences ($p<0.05$) in exfoliated cells from the lateral border of the tongue (keratinized epithelium). This effect was more pronounced when patients were exposed to a repeat radiograph; however, an increased risk of irreversible tissue damage or genotoxic effect was not implied. The present study was in accordance with the study conducted by Pai et al. and Da Silva et al.

In our study, while comparing total Mni percentage in the buccal mucosa and keratinized gingiva, there was no statistically significant increase ($p= 0.1201$) before and after exposure ($p= 0.7190$). Comparison with other studies was not possible because there are no studies available pertaining to Mni percentage in the buccal mucosa and keratinized gingiva and therefore, further research is required to find any correlation between the buccal mucosa and keratinized gingiva. Detection of micronuclei and their assay is nowadays becoming a field of research interest for prevention and treatment of cancer. These micronuclei if properly identified can turn out to be an important biomarker for screening and prediction of the patients with potentially malignant lesions. These can also prove to be very useful for assessment of the risk in patient's ongoing treatment for invasive cancers.

CONCLUSION

In the present study, the genotoxic effect of radiation exposure from panoramic radiography showed a statistically significant increase in the MN frequency in buccal epithelial cells that can increase chromosomal damage and induce apoptosis. Our findings of the micronucleus test in indicating genotoxicity add valuable information to complete the overall elucidation of the effect of radiation exposure for diagnostic purposes. Thus, panoramic dental radiography should be cautiously used only when necessary and any retakes that may increase radiation dose and hence, increase the cytogenetic damage should be avoided.

This study thus provides an insight for the early diagnosis of cancer, even when it is not clinically evident and is sensitive even for low doses of radiation exposure, but further research including larger sample sizes and strict adoption of the scoring criteria would be desirable to confirm the conclusion of this study.

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