Genotoxic Effects of Dental Panoramic Radiograph in Children

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*This **study** was completed to evaluate chromosomal damage (micronucleus) and cellular death in exfoliated buccal mucosa cells taken from healthy children following exposure to panoramic radiation during dental radiography. **Method:** Twenty children who underwent panoramic dental radiography for diagnostic purposes were included. Cytological preparations were stained with Feulgen stain, identified under light microscopy. Micronuclei, apoptotic nuclear alterations (condensed chromatin, karyorrhexis, pyknosis) and necrosis (karyolysis) were scored. **Results** showed no statistically significant differences in children's micronucleated oral mucosa cells before and after panoramic dental X-Ray exposure. On the other hand, there was a statistically significant increase in nuclear alterations closely related to genotoxicity such as condensed chromatin, karyorrhexis and pyknosis, while karyolysis of oral mucosal cells did not show significant increase after panoramic X-Ray exposure. **Conclusion:** Dental panoramic radiography may not be a factor that induces chromosomal damage, but is able to promote genotoxicity in children.*

Keywords: *Micronucleus test, Buccal mucosa cells, Panoramic X-Ray, Children.*
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INTRODUCTION

X-Rays are important tools for diagnosis in medical and dental practice; however, it is well known that X-Rays can induce cytotoxic effects and chromosomal damage.[1-5] Panoramic radiography is recommended by dentists when the evaluation of all teeth is necessary to complement the clinical examination, since it is considered less harmful than several periapical radiographs. It is largely known; however, that there is no safety in radiation doses and that the biological effects of the exposures received would be accumulated thorough the time.[6] Children, who are still developing, express increased susceptibility to environmental hazards, chronic infection and inflammation, dietary factors and long-term medication because of differences in the uptake, metabolism, distribution and excretion of chromosomal damaging agents.[7]

The micronucleus (MN) test performed in lymphocytes or in exfoliated cells is a very reliable assay to evaluate human genetic damage.[8,9] This test has different applications, and is particularly useful for biomonitoring human populations under exposure to chemical and physical mutagenic agents.[10-15] In ionizing radiation studies, this test has been used to assess genetic damages after occupational exposure to X-Rays,[15,16] to measure cell radiosensitivity[17-19] to study the persistence of chromosomal aberrations in dividing cells[20] and to detect genotoxic effects of photon radiation.[21] According to Tolbert *et al,*[22] the specificity of the test to detect genotoxic and cytotoxic effects is increased by recording other degenerative nuclear alterations indicative of cell death, besides the micronucleus.

Cytogenetic methods have been most extensively used for the biological monitoring of populations exposed to known mutagens and carcinogens.[23] A great deal of interest was raised by the application for this purpose of the micronucleus test to uncultured exfoliated cells.[24] MNs observed in exfoliated cells are not induced when the cells are at the epithelial surface, but when they are in the basal layer. The presence of micronuclei is indicative of chromosome loss or fragmentation occurring during previous nuclear division.[25] MN is formed during the metaphase/anaphase transition of mitosis (cell division). It may arise from a whole lagging chromosome (aneugenic event, induced by agents that affect the spindle apparatus leading to chromosome loss) or an acentric chromosome fragment detaching from a chromosome after breakage (clastogenic event, induced by substances that cause chromosome breakage) which do not integrate in the daughter nuclei.[26] Thus, Micronuclei consisting of whole chromosomes (centromere positive, larger MN) indicate aneuploidogenic genotoxic effects. Micronuclei, however, which contain chromosome fragments (centromere negative, smaller MN) base on clastogenic genotoxic effects.[21]

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In human cytogenetic studies, some confounding factors must be considered. Viruses, alterations in the immune system, failures in the DNA repair system, and inter-individual variations have been associated with increased frequencies of chromosome aberrations.[27]

Ionizing radiation is a well-known mutagen and carcinogen in the human population. Thus, the present investigation was performed in order to detect the possible genotoxic effects which may be induced by radiation emitted during the exposure of children to low dose diagnostic panoramic radiographs, using a sensitive analysis.

MATERIALS AND METHOD

Sample

Twenty healthy children with the signed informed consent approved by the faculty ethics committee were included in this study (12 boys and 8 girls between the ages of 5-13 years) as outpatients at Department of Pedodontics, Al-Azhar University for Girls'. Panoramic dental radiography examinations were requested by the dentist and were performed at Oral Medicine, Periodontology and Radiology department using Orthopantomograph (OP100, Finland) with the following setting: 70 kv, 24 mA 16 sec, 110 mGycm.[2] The entrance dose was 0.08 R. A careful detailed medical and dental history was taken from each selected patient. None of the children evaluated was exposed to other known genotoxic agents.

Cell Collection

Prior to buccal cell collection, children rinsed their mouths thoroughly with water to remove any unwanted debris. Exfoliated buccal mucosa cells, which are target cells for dental radiography, were obtained by scraping the right/left cheek mucosa with a wooden spatula immediately before the X-Ray exposure and after 10±2 days from each child. The cells were collected in a sample bottles containing 20 ml of buffer solution (0.1M EDTA, 0.01 Tris–HCl and 0.02M NaCl, pH 7).

Cytological Preparations

The collected solutions were centrifuged (800 rpm) for 5 min, cell suspension were dropped onto preheated (55°C) slides and allowed to air dry for 15 min on a slide warmer. The slides were fixed in 80% cold methanol for 30 min, air-dried overnight at room temperature, and stored at −20°C until use. The slides were stained with Feulgen (DNA specific stain) and examined under a light microscope to determine the frequency of micronucleated cells.

The Feulgen technique consists of two steps according to Nersesyan *et al.*[28] The fixed slide is treated for 8-10 min with 1N HCl in a water bath or oven at 60°C. Afterwards, the slide is immediately transferred into Schiff's reagent at room temperature (for at least 30 min or until the tissue stains deep purple). The slide is then squashed in acetocarmine or aceto-orcein. Acid hydrolysis removes purin bases from the DNA, thereby unmasking free aldehyde groups. The aldehyde groups then react with Schiff's reagent, which results in the purple staining. RNA is not hydrolyzed by the HCl treatment and, thus, the reaction is DNA-specific.

Cytological Scoring Criteria

The various distinct cell types (cells with micronuclei, condensed chromatin, karyorrhexis, pyknosis and karyolysis) scored in the buccal epithelial cells were determined based on criteria described by Tolbert *et al.*[29] These criteria are intended to classify buccal cells into categories that distinguish between "normal" cells (Figure 1A) and cells that are considered "abnormal," based on their aberrant nuclear morphology. These abnormal nuclear morphologies are thought to be indicative of chromosomal damage and/or various stages of morphogenetic or toxicity-induced cell death. Cells with micronuclei are characterized by the presence of both a main nucleus and one or more smaller nuclei called micronuclei. A MN must be (i) less than one-third of the diameter of the main nucleus, may range between 1/3 and 1/16 the diameter of the main nucleus; (ii) on the same plane of focus; (iii) have the same color, texture and refraction as the main nucleus; (iv) have a smooth, oval or round shape; (v) be clearly separated from the main nucleus (vi) must be located within the cytoplasm of the cells[30] (Figure 1B). Micronuclei were scored only in basal and differentiated cells with uniformly stained nuclei.

Based on previous criteria of Tolbert *et al.,*[29] the condensed chromatin cells revealed nuclei with regions of condensed or aggregated chromatin exhibiting a speckled or striated nuclear pattern. In these cells it is apparent that chromatin is aggregating in some regions of the nucleus while being lost in other areas. When chromatin aggregation is extensive the nucleus may appear to be fragmented, (Figure 1c). These cells may be undergoing early stages of apoptosis. The karyorrhectic cells showed more extensive appearance of nuclear chromatin aggregation (relative to condensed chromatin cells) leading to fragmentation and eventual disintegration of the nucleus, (Figure 1d). Both condensed chromatin and karyorrhectic cells did not score for micronuclei in the assay. The pyknotic cells showed a small shrunken nucleus, with a high density of nuclear material that is uniformly but intensely stained. The nuclear diameter is usually one to two-thirds of a nucleus in normal differentiated cells (Figure 1e). The karyolytic cells showed completely depleted of DNA and apparent as a ghost-like image that has no Feulgen staining. These cells thus appear to have no nucleus[31] (Figure 1f).

Cytological analysis

For each individual a minimum of 1000 buccal cells were studied both before and after X-Ray exposure. The total number of cells was automated counted by using Leica image analysis computer system at Oral and Dental Pathology Department, Faculty of Dental Medicine, Al-Azhar University for Girls'. Automated slide scanning is generally performed according to Varga *et al.*[32] by moving the slide with reference to the fixed objective lens of the microscope in a regular meander-like pattern, leaving no gaps between the

image fields. Because of speed considerations, image acquisition is done at the lowest possible optical magnification that still allows resolution of the features of interest (to detect and count nuclear alterations a 20X objective, giving a final magnification of 200X, is used). Each field of view is captured and analyzed for the presence of analyzable objects. If cells of interest are detected within a field, they are further analyzed and stored in an image gallery along with their position and feature data. Micronuclei and other nuclear alterations were confirmed by observing them in oil immersion at 1000X magnification. Computerized image analysis has the advantage of fast acquisition of results, which allows the analysis of large numbers of slides and the exclusion of individual scoring skills.

Statistical analysis

Data were presented as frequencies and percentages. Chi-square (x^2) test was used for comparisons between changes after exposure. The significance level was set at $p \leq 0.05$. Statistical analysis was performed with SPSS 16.0® (Statistical Package for Scientific Studies) for Windows.

RESULTS

In the present study, the genotoxic effects of X-Ray exposure during panoramic dental radiography were evaluated immediately before and the tenth day after exposure. The total number of cells analyzed before and after exposure was 27025 and 28665, respectively. The cells with micronuclei showed presence of both a main nucleus and one or more smaller nuclei. The frequency of the MN cells was higher after panoramic X-Ray exposure; however, statistical comparison of micronucleus occurrences did not show significant difference ($p < 0.792$). The frequencies of condensed chromatin, karyorrhexis and pyknosis were significantly higher after panoramic X-Ray exposure ($p < 0.001$). The frequencies of karyolysis is higher after panoramic X-Ray exposure; however, statistical comparison between frequencies of karyolytic cells before and after panoramic X-Ray exposure showed no significant difference ($p < 0.099$).

Table (1) presents the total number of micronuclei and other nuclear alterations observed before and after X-ray exposure. Table (2) presents the frequencies, percentages and results of chi-square test for the comparison between micronuclei and other nuclear alterations before and after X-Ray exposure.

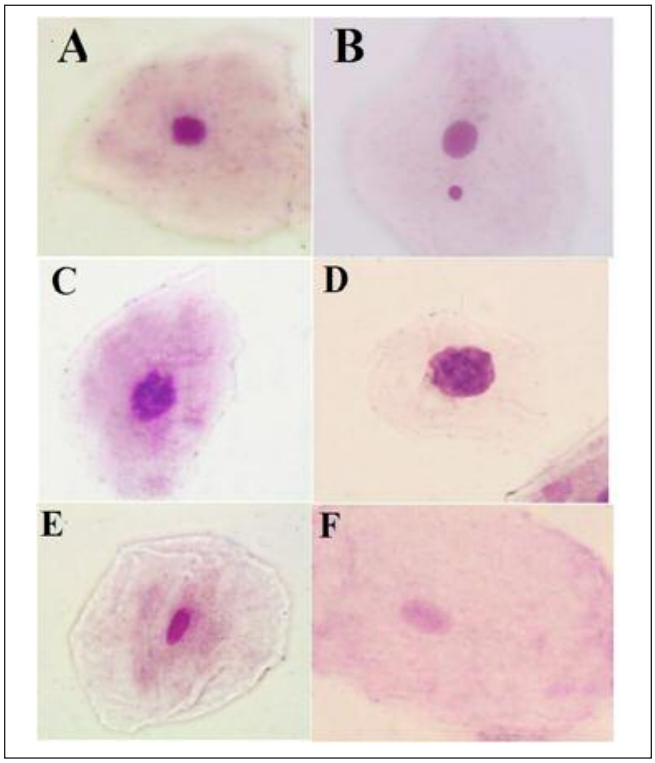

Figure 1: Microscopic views of human buccal cells showed a) normal cell, b) micronucleated cell, c) cell with condensed chromatin, d) Karyorrhectic cells e), Pyknotic cells, f) Karyolytic cells (Feulgen stain, original magnification 630X).

Table 1. Total number of micronuclei and other nuclear alterations before and after X-Ray exposure

Sample	Number of cells	Micronuclei	Condensed chromatin	Karyorrhexis	Pyknosis	Karyolysis
Before exposure	27025	10	65	120	45	20
After exposure	28665	12	210	325	175	35

Table 2. Frequencies, percentages and results of chi-square test comparing micronuclei and other nuclear alterations before and after X-Ray exposure

Cellular Alterations	Before exposure		After exposure		*P*-value
	Frequency	%	Frequency	%	
Cells with micronuclei	10	0.04	12	0.04	0.792
Condensed chromatin cells	65	0.2	210	0.7	<0.001*
Karyorrhectic cells	120	0.4	325	1.1	<0.001*
Pyknotic cells	45	0.2	175	0.6	<0.001*
Karyolytic cells	20	0.1	35	0.1	0.099

**: Significant at* $p \leq 0.05$

DISCUSSION

The epithelial cell kinetics is important in the interpretation of the results obtained with the micronucleus test. Chromosomal damage leading to micronucleus formation occurs in dividing cells from the basal layer of oral epithelium, but it is only observed later in exfoliated cells after the differentiation. The turnover of this epithelium is rapid (from 7 to 16 days),[33,34] and thus the maximal rate of micronucleus is expected between 1 and 3 weeks subsequent to the exposure to a genotoxic agent.[35-37] Superficial buccal cells are pulled continuously and replaced by cell division of the basal stem

cell. When the basal stem cells divide, damaged and fragmented chromosomes can lag during mitotic division and appear in the cytoplasm of the daughter cells as a small nuclear particle, termed micronucleus.[34,38-39] Therefore, the design of the present study required a time period of 10±2 days which is the period for their replacement with basal cells from squamous epithelium to detect the maximum effect of radiation exposure

Studies about MN have been carried out in populations exposed to ionizing radiation, smokers, gas station workers, workers exposed to pesticides, styrene, ethylene dioxide, polycyclic aromatic hydrocarbons, and patients with different kinds of epithelial cancers (such as oral, esophageal, lung, bladder, etc.).[30,40-42] Micronucleated cell indexes might reflect genomic instability.[43-44] Thus detection of an elevated frequency of micronuclei in a given population indicates an increased risk of cancer.[45]

Micronucleus trials in children with non-neoplastic and neoplastic lesions revealed increased micronuclei frequency compared with healthy controls.[46] In this regard, Ribeiro *et al,*[47] compared the micronucleus frequency and cytotoxicity after X-Ray exposure in order to know if children are more susceptible to this potential harm. Their results demonstrated that the micronucleus frequency did not increase following exposure to ionizing radiation when compared with adults. This also occurred with the cytotoxicity. Taken together, it is assumed that children are not more susceptible to the noxious activities induced by X-Rays when compared to adults.

Because micronuclei are assumed to be expressed in dividing cells that contain chromosome fragments and/or whole chromosomes that are unable to migrate to the spindle poles during mitosis, thus, the micronucleus test is considered appropriate, noninvasive method, a suitable biomonitoring approach to assess chromosome damage in children following X-Ray exposure. Micronuclei Scoring can be performed relatively easily on exfoliated epithelial cells, without extra *in vitro* culture.

Epithelial buccal cells to evaluate the effects of X-ray of panoramic radiograph are more appropriate because they are target cells which can be easily, simply and routinely analyzed. This approach has several advantages because epithelial cells of buccal mucosa are the target cells for dental X-Ray exposure. In addition, epithelial cells are highly proliferative and are the origin of more than 90% of all human cancer.[12] Unlike what has been reported in lymphocytes, there are no consistent sex or age effects on the frequency of MN in exfoliated cells. Although the spontaneous frequencies of MN are similar in all types of exfoliated cells, these levels can increase significantly at different sites in response to specific exposures.[35] Therefore, the application of micronucleus test in epithelial cells is considered to be a sensitive tool to bio monitor the genetic damage in human population.[12,33-34,38,48-49]

Variability of MN assessment arises from intra- and inter-individual variability, and population variability, as well as variations characteristic of different cell types. The average reported healthy population MN frequency is 1–3 *per* 1000 cells, with no significant variation between different types of exfoliated cells.[35] Repeated scoring of MN in epithelia from the same individuals showed variation between 30 and 102.9%.[38,50] This may be considered a measure of intra-individual variability, which reflects random variation in the observation of relatively rare events in a limited number of trials. When spontaneous MN frequencies were compared in different healthy individuals, up to a 17-fold difference was observed, possibly reflecting genetic and nonspecific exposure differences.[36,51] The causes of this inter-individual variability are unclear at this time and deserve further investigation.

This study showed that the micronucleus frequencies were not statistically significantly different ($p < 0.792$) before and after X-Ray exposure. Such findings are in line with studies of several authors.[6,47,52] These results contrast with the observations of others who reported higher rates of chromosomal aberrations subsequent to X-ray exposures using other test systems.[53-55] Bio-monitoring studies of populations exposed to X-Rays are quite difficult and rather specific because each population is exposed to different doses of radiation. This could explain why some studies have shown an increase in genetic damage in populations exposed to X-Rays. Taken together, this study assumed a lack of clastogenic and/or aneugenic effects related to panoramic dental radiography in children.

To monitor genotoxic and cytotoxic effects (apoptosis and necrosis, respectively), the frequencies of cells showing condensed chromatin, karyorrhexis, pyknosis and karyolysis were evaluated in this experimental design. Despite the lack of cytogenetic damage, the frequencies of nuclear alterations indicative of apoptosis (condensed chromatin, karyorrhexis and pyknosis) in these results were statistically significantly ($p< 0.001$) after the exposure. Similar results have been reported by others.[6,47,56-57] Karyorrhectic cells may be undergoing a late stage of apoptosis.[31] Apoptosis is a fundamental biological process, which is genetically controlled and required for both normal development and tissue homeostasis.[58] These results showed that the panoramic dental radiography induced the apoptotic response, which probably interfered with micronucleus induction. Similar results are described by Torres-Bugárin *et al.*[42] They verified significantly higher frequencies of micronucleus in controls when compared to patients *undergoing* anti-neoplasic chemotherapy probably as a consequence of the cytotoxic effects of the therapy on buccal mucosa cells.

Karyolytic cells may represent a very late stage in the cell death process.[31] Karyolysis did not statistically significantly increase ($p < 0.099$) after the radiation exposure, suggesting that the cellular response to X-Rays does not include a cytotoxic effect that leads to necrosis. However, in the post-exposure results, a significant higher number of nuclear alterations characterized by disruption of nuclear contour and chromatin shrinkage were found which may result from cytotoxicity. If true, this will be an additional factor interfering in the micronucleus occurrence, once it is known that

such frequency generally declines as the concentrations of genotoxic chemicals reach toxic levels.[21]

CONCLUSIONS

The results of the present study suggest that X-Rays can induce genotoxic effects in oral mucosa cells. In this regard, panoramic dental radiography should be used only when necessary because it can induce cellular death. This study also confirms the usefulness of the micronucleus assay and the greater sensibility of Tolbert´s *et al*.[22,28] protocol in biomonitoring studies conducted in children, emphasizing their great sensitivity even to exposure to low doses of environmental agents. Further studies in children would be beneficial.

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