RESEARCH ARTICLE

Evidence of genotoxicity and cytotoxicity of X-rays in the oral mucosa epithelium of adults subjected to cone beam CT

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Objectives: To assess cytological evidence of genotoxicity and cytotoxicity of X-rays in oral exfoliated cells of adults subjected to partial and total cone beam CT (CBCT) (stitching module) by means of micronuclei frequency, associated with counting of degenerative nuclear alterations (pyknosis, karyolysis, karyorrhexis, buds and broken eggs), besides comparing the partial and total CBCT (stitching module) in search of possible differences in the nature and/or intensity of the effects.

Methods: 29 adults who were referred to total or partial CBCT were selected. All CBCT were performed with a Carestream CS 9000 3D scanner (Carestream Health Inc., Rochester, NY). Material collection was done immediately before CBCT and 10 days later, by scraping the left and right cheek mucosa with a plastic spatula. Statistical analysis was performed using the Wilcoxon test (paired data), at a significance level of 5%.

Results: The statistically significant difference was noted in the frequency of micronucleated cells for both partial and total acquisition ($p = 0.008$ and $p < 0.001$, respectively). Regarding to cytotoxicity, there was a statistically significant difference for both partial and total acquisition ($p < 0.001$ and $p < 0.001$, respectively).

Conclusions: The partial and total CBCT seems to offer risks of inducing genetic damage. In addition both forms of CBCT acquisition have promoted the induction of cytotoxic nuclear alterations.

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Introduction

Cone Beam Computed Tomography (CBCT) is regarded as an excellence examination in dentistry, after having demonstrated its benefits, quality and diagnostic accuracy in recent years, allowing greater reliability in diagnosis and planning for the dentist.¹⁻³

Unlike conventional radiographs, which project all the structures crossed by X-rays in a single plane, the

CBCT shows the structural relationships in depth, allowing the visualization of all the structures in multi-planar reconstructions with a considerable definition, mainly of the mineralized tissues, such as bone.³⁻⁵ Therefore, before the limitations in obtaining information for the diagnosis with the use of conventional radiographs, the three dimensional (3D) images attracted the interest of dentists.⁵

There are equipment with varying field of view (FOV) sizes. The FOV can vary from a few centimeters

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in height and transverse diameter to those that allow reconstruction of the entire head and neck region. FOV selection should be related to the planning and diagnosis required, as this parameter is directly associated with the dose of radiation to the patient.^{3,6,7} Large FOVs are used most often for orthognathic surgery and/or orthodontic treatment. However, for most other situations, a smaller FOV is adequate. In addition to being more accessible, producing better quality images, higher contrast and lower artefact production, it reduces patient exposure to radiation.^{3,8}

There is also a new form of 3D acquisition, known as 3D stitching (Carestream Health Inc., Rochester, NY). This process automatically combines three small FOV volumes to construct a larger, composite 3D image that is needed for a wider region of examination. The benefits of the stitching module include a broader range of applications, accessibility, flexibility and improved workflow.⁸ However, it is noted that this horizontal composition results in the scanning of some overlapping regions twice, thus doubling the dose of radiation administered to those regions.²

Although CBCT provides great benefit to the practice of dentistry, their use must be indicated by clinical justification, since even a low dose of X-rays is capable of causing cytotoxic effects and DNA damage.^{9–11} The frequency of micronuclei has been used to verify genotoxic effects in human tissues, as well as the degree of exposure and extent of damage that an environmental agent has caused in DNA.^{12–15} Micronuclei are fragments or whole chromosomes that were lost during cell mitosis owing to a clastogenic or aneugenic event.¹⁶ X-rays are clastogenic agents and induce the formation of micronuclei, in addition to other nuclear alterations.^{17–19}

The oral mucosa epithelium can be considered the tissue of choice for the analysis of the effects of X-rays, because it is under direct radiation exposure at CBCT examination and in that way it is the primary target for radiation-induced damage and, moreover, this tissue has the advantage of rapid and easy sampling by scraping the buccal mucosa.²⁰ According to Sarto *et al*,¹⁶ these effects can be studied by means of exfoliative cytology on a liquid basis, because it is simple to perform, low cost and non-invasive, in addition to optimizing the microscopic analysis of the blades with the highest number of individual epithelial cells desquamated.

For the analysis of the effect of X-rays on epithelial tissues, it is indicated the use of the micronucleus test combined with the count of degenerative nuclear alterations.^{21,22} Nuclear alterations in exfoliated buccal cells have been considered as markers of cytotoxic effects, representing different degenerative and/or adaptive phenomena of cell death.^{22,23} Therefore, this association increases the specificity and sensitivity of the biomonitoring of populations exposed to genotoxic and cytotoxic agents, including X-rays.

It has been reported that the use of conventional radiographs, such as panoramic radiography and

lateral tele radiography, may produce cytotoxic effects and cause DNA damage,^{9,11,18,20,24} but there are few studies that discuss these effects produced by the use of CBCT.^{1,19} Until then, there is not analysis in the literature of the effect caused by the methodology of CBCT acquisition by the stitching module.

Knowing that micronucleus frequency is considered a reliable tool for the early detection of systemic malignancy conditions, since genomic instability seems to be associated with cancer risk and progression,¹⁷ as well as that cytotoxic agents interfere in the molecules closely involved in cell growth and death,²⁵ this study aimed to contribute to the increased knowledge of the action of X-rays on human tissues through analysis the genotoxicity and cytotoxicity of X-rays on buccal epithelial exfoliated cells of adults submitted to CBCT, also comparing the damage caused by partial and total CBCT (stitching module).

Methods and materials

Subjects and ethical considerations

The participants of this study consisted of 29 healthy adults (12 males and 17 females), with a mean age of 45.8 ± 12.5 . All CBCT scans were taken at the same private establishment of dental radiology in Aracaju, SE, Brazil. Information and individual characteristics of the participants were collected and included gender, age, habits and exposure to genotoxic agents. All subjects were non-smokers, did not use alcohol and/or mouthwash. All CBCT were requested by a dentist and was performed with a Carestream CS 9000 3D scanner (Carestream Health Inc., Rochester, NY).

This scanner is used on dentomaxillofacial region, in which two different protocols were applied, depending on the dentist's request: for those who were requested partial CBCT, the protocol of 70 kV, 10 mA and 10.8 s was applied, under a minute voxel size of 200 μ m and FOV of 50 $\times$ 37 mm. The site of choice was the posterior region of the right or left jaw for partial CBCT. For total CBCT (stitching mode), the protocol of 70 kV, 10 mA and 32.4 s was used, under a minute voxel size of 200 μ m and FOV of 80 $\times$ 37 mm. The region chosen for total CBCT was the mandible.

Free and informed consent was obtained from the participants and the research was approved by the Research Ethics Committee with human beings of the University Hospital of the Federal University of Sergipe-UFS, Brazil (CAAE: 53233716.5.0000.5546).

Collection of cells and slide preparation

Exfoliated oral mucosa cells were collected immediately before CBCT exposure and after 10 days. After rinsing the mouth with water, cells were collected by scraping the right/left cheek mucosa with an Ayre plastic spatula (Stra Medical, Balneário Camboriú, SC, Brazil). Cells were transferred to a tube containing fixing solution

(Stra Medical, Balneário Camboriú, SC, Brazil), homogenized for 30 s at speed four (NI 1059-Novainstrumentos Ltda., Piracicaba, SP, Brazil), centrifuged for 10 minutes at 1000 rpm (Baby I 206-FANEM, Guarulhos, SP, Brazil), and finally dropped onto precleaned slides. The air dried slides were stained using Papanicolaou technique.

Cytological analysis

The slides were examined in a random order by a blind examiner previously calibrated by an experienced oral pathologist. He has more than 25 years of experience as an oral pathologist and a cytopathologist. A transmitted light microscope (Olympus CX31, São Paulo, SP, Brazil) was used, at 400× magnification to determine the frequency of micronucleated buccal mucosa cells and other nuclear alterations. A total of 1000 cells were analysed from each patient for each sampling time (before and after CBCT exposure). For analysis of DNA damage (genotoxicity), the micronucleated cells were scored according to the criteria described by Sarto *et al.*¹⁶ For cytotoxicity, the following nuclear alterations were considered, as described by Tolbert *et al.*²¹: pyknosis, karyolysis, karyorrhexis, bud and broken eggs. Results were expressed in percentages (%).

Statistical methods

First, the Shapiro-Wilk test evaluated the sample distribution. The Wilcoxon test (paired data) was used to compare the frequencies of nuclear changes associated to genotoxicity and cytotoxicity before and after CBCT exposure. The level of statistical significance was set at 5%.

The reliability of the evaluation was verified by reanalysis after 30 days of 20% of the samples before and after CBCT exposure. The κ test was applied to investigate the agreement between the two evaluations. For these purposes, IBM Statistics software, v. 22.0 (IBM Corporation, Armonk, NY; formerly SPSS Inc., Chicago, IL) was used.

Results

According to the result of κ test, which coefficient resulted in a value of 0.86, it was verified that there was almost perfect agreement²⁶ between the evaluations of the sample, regarding the identification of the structures corresponding to both cytomorphological signs of genotoxicity (micronucleated cells) and cytotoxicity (pyknosis, karyolysis, karyorrhexis, bud and broken eggs).

All the analysed cytological smears presented different nuclear alterations in various proportions and frequencies, before and after the radiographic procedures. Among the alterations analysed, pyknosis was the most frequent cytopathological finding, being found especially in superficial cells, keratinized or not.

The buds and broken eggs, on the other hand, were the least alterations found in the smears of jugal mucosa. Figure 1 shows an iconographic representation of the cytomorphological findings observed in the present study.

As can be seen in Figure 2a, there was statistically significant difference in micronucleated cell frequency before and after exposure to ionizing radiation resulting from partial CBCT ($p = 0.008$). Analysis of cytological samples from subjects undergoing total CBCT (Figure 2b) revealed a significant increase in micronucleated cell frequency ($p < 0.001$).

Figure 3 expresses the frequency of pyknosis, karyolysis, karyorrhexis, buds and broken eggs, cytomorphological findings interpreted as suggestive of cytotoxicity in the analysed jugal mucosa smears. A significant increase of these nuclear alterations can be observed for both partial CBCT ($p < 0.001$) and total CBCT ($p < 0.001$).

Discussion

Micronucleus test in exfoliated buccal cells has been widely used in the monitoring of individuals or populations exposed to genotoxic or mutagenic events since the 1980s.^{12,13,15,27–29} The limited cost, speed and simplicity of methodological procedures, ease of analysis and the precision obtained in the data, especially when registering large numbers of cells, determine the popularity of this non-invasive method.^{19,30} In view of this, the advantage of the micronucleus assay to elucidate the effects of genotoxic and cytotoxic agents directly on a target tissue is clear.

In 1992, a protocol that combined the micronucleus count with the analysis of other nuclear anomalies such as pyknosis, karyolysis, karyorrhexis, buds and broken eggs was proposed, for being considered phenomena that can occur during cell death with DNA damage.²¹ From then on, this has been the test used in several researches, which justified your achievement in the present study.^{10,19,20,24,29,31}

The option to analyse nuclear alterations suggestive of genotoxicity and cytotoxicity in the oral epithelium to monitor the damage caused by ionizing radiation was based on the fact that this tissue was under direct X-ray exposure during the CBCT.¹⁹ The collection of cells performed immediately prior to X-ray exposure and 10 days later was based on previous studies with similar methodology.^{1,9,10} This period of 10 days was adopted because the damage that determines the formation of micronuclei and other nuclear alterations occurs in the basal layer of the epithelial tissue. Thus, only after cell turnover of the oral epithelium, approximately 21 days, these cells reach the surface of the mucosa, where they exfoliate.^{22,32} Therefore, this period allowed the basal layer exposed to the radiation to mature and then be easily and effectively collected by cytological swabbing techniques when suffering spontaneous exfoliation.

Figure 1 Nuclear alterations observed in cytological smears stained by the Papanicolaou technique of patients submitted to ionizing radiation during performance cone beam CT. (a, b) micronucleus (straight arrows); (c, d) pyknosis (dotted straight arrows); (e, f) karyolysis (#); (g, h) karyorrhexis (*); (i, j) broken eggs (curved arrows); (k, l) nuclear buds (dotted curved arrows).

Studies have shown an increase in micronucleus frequency in oral cells associated with smoking,²⁷ frequent alcohol consumption^{27,33} and continued use of oral mouthwash.³⁴ Therefore, to obtain an accurate

data analysis, smokers, alcoholic or users of oral mouthwashes were excluded from the sample. As in similar studies,^{9,25} this exclusion criteria were adopted to avoid such factors influencing the results because the observed

Figure 2 Frequency of micronucleated cells observed in the cytologic smears of jugal mucosa of patients submitted to cone beam CT partial (a) and total (b). Data expressed as median (min to max). * $p < 0.01$; statistically significant difference between groups (Wilcoxon test).

Figure 3 Frequency of cells with other nuclear alterations (pyknosis, karyolysis, karyorrhexis, buds and broken eggs) observed in the cytologic smears of the jugal mucosa of patients submitted to cone beam CT partial (a) and total (b). Data expressed as median (min to max). $^{**}p < 0.001$; statistically significant difference between groups (Wilcoxon test).

effects could be incorrectly attributed to the variable investigated.¹³ In addition, each patient was considered as their own control. Thus, any effect of other genotoxic agents must have been present in the first cell count. Therefore, the differences between the first and second counts can be attributed to radiation.^{19,35}

Genomic instability seems to be associated with cancer risk and progression. Thus, the detection of a high frequency of micronuclei in a given population indicates an increased risk for the onset of cancer, being considered a reliable tool for the early detection of systemic conditions of malignancy.¹⁷ The results of the present study demonstrated that the frequency of micronuclei was statistically significant difference before and after partial CBCT. Similar studies have shown different results.^{1,19} However, these authors used different CBCT units, kilovoltage (kV) and milliamperage (mA). There is not analysis in the literature of the effect caused by the methodology of CBCT acquisition by the stitching module. In addition, partial CBCT presented with lower frequencies of micronuclei in relation to total CBCT. These data seem to attest that there are significant risks of induction of genetic alterations after radiation at the dose used in the present study.

In the comparison between before and after the acquisition of total CBCT, a significant increase of micronucleated cells was also observed. During the total acquisition by the stitching mode, the automatic incorporation of three volumes of small FOVs is performed to construct a larger one, forming a 3D image of a wider region. Although this process brings benefits to the dentist and patient,⁸ it is noted that this horizontal composition results in the scanning of overlapping regions twice, thus doubling the dose of radiation administered to these regions.^{2,8}

The interpretation of the real clinical significance of these findings, and their relationship to a probable carcinogenic potential, should be made with parsimony, since the magnitude of the effects of radiation exposure depends primarily on the dose and frequency

used, as well as on the individual's ability to repair such damage.³⁶ In this sense, it should be considered that the mean effective doses for the partial (25 μ Sv) and total (44.5 μ Sv) CBCT scans^{37–39} were well below the effective dose limit in 1 year recommended by the International Commission on Radiological Protection (1 mSv).⁴⁰ Thus, as long as the request for CBCT acquisitions is performed in a rational manner, respecting the indication limits and the maximum annual dose of X radiation, it is possible to suggest that the accomplishment of this complementary examination would have practically inexpressive repercussions for the patient.

To monitor cytotoxic effects, the frequencies of pyknosis, karyolysis, karyorrhexis, buds and broken eggs were evaluated. The results obtained in the present study demonstrated that both partial and total CBCT (stitching mode) were able to induce nuclear alterations suggestive of irreversible cell injury and, therefore, indicative of cell death. Despite the cytotoxicity of X-rays and their ability to induce biomolecular changes that lead to cell death owing to biochemical phenomena associated with necrosis and apoptosis, have been well established since the 1990s,⁴¹ the precise mechanisms behind such effects cytotoxic agents are still poorly understood.¹ However, similar results have been described by other investigators,^{1,19} which seems to attest the cytotoxicity of this form of radiation, corroborating the findings of the present study. These data ratify the premise that, despite its many advantages as a complementary diagnostic tool, imaging procedures should be performed under precise clinical indications and under conditions that allow the radioprotection of structures other than those to be irradiated.

Therefore, it is suggested that other studies should be carried out with larger samples to minimize the effect of variations in the symmetry of data distribution and to more clearly evaluate possible differences in the percentage of increase of cells with genetic damage in total and partial CBCT.

In conclusion, from the present study it is possible to suggest that partial and total CBCT seems to offer risks of inducing genetic damage. In addition, both forms of CBCT acquisition have promoted the induction of

cytotoxic nuclear alterations. However, further studies are still needed to investigate the clinical repercussions of these findings and their real impact on patients' health.

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