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ORIGINAL ARTICLE

Comet Assay Assessment of DNA Damage in Buccal Mucosa Cells Exposed to X-Rays via Panoramic Radiography

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ABSTRACT

Ionizing radiation (IR) presents a risk to human health via DNA damage even when administered at low doses, such as those used in panoramic radiography. **Objectives:** This study used the comet assay to assess DNA damage in buccal mucosa cells consequent to X-ray radiation from panoramic radiography. **Methods:** Twenty participants were recruited from among patients who underwent panoramic examinations at Prof. Soedomo Dental Hospital, Universitas Gadjah Mada, and divided into two groups of 10. Buccal mucosa cells were collected from all participants before exposure to IR and at 30 min or 24 h after exposure in groups 1 and 2, respectively, and subjected to a comet assay to assess DNA damage. Assay output images were analyzed using OpenComet software. Double-strand breaks (DSBs) were assessed by comparing the percentages of tail DNA in output images obtained before and after X-ray exposure. **Results:** A statistically significant ($p=0.014$) increase in the percentage of tail DNA was observed at 30 min after exposure, but not at 24 h ($p=0.29$). **Conclusion:** Panoramic X-ray radiation may induce DSBs in human buccal mucosal cells within 30 min after exposure.

Key words: buccal mucosa, comet assay, DNA damage, panoramic radiography

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INTRODUCTION

The human body is constantly exposed to radiation in the surrounding environment, both from natural radiation sources, such as atmospheric cosmic rays, rocks, soil, uranium, and radon, and artificial sources, such as medical equipment.¹ According, radiation cannot be ignored as a potential cause of DNA damage. Each year, the human body is exposed to approximately 3.1 mSv of natural radiation, which is comparable to the average exposure from medical devices. Regarding medical sources of radiation, imaging devices and technology comprise the largest fraction.

Currently, dental X-ray radiography examinations contribute to 0.26% of the lifetime exposure to radiation.² The doses used in dental radiography are relatively low—approximately 0.322 μ Sv for periapical radiography and 2.7–38 μ SV for panoramic radiography¹—and remain below the radiation exposure dose limit required by the International Commission on Radiological Protection (ICRP; 0.3 mSv/year).³

Nonetheless, even low doses of X-ray radiation can induce adverse biological changes in living tissues.⁴ Thus, it has been conservatively assumed that the biological damage caused by ionizing radiation is directly related to radiation exposure, regardless of the magnitude of the dose. In other words, all radiation doses are considered harmful.⁵

The effects of panoramic radiography have been widely studied,^{1,6,7} leading to the findings that panoramic radiography causes chromosomal damage, nuclear changes, and cell death. However, no previous reports have addressed the DNA-damaging effects of radiation from panoramic radiography in buccal mucosa cells. The determination of DNA damage in a cell is difficult, as the degree of damage is relatively small and the measurements tend to involve a limited number of samples.

Recent studies have implemented the comet assay to measure DNA damage.⁶ This assay, which is sensitive enough to measure injuries to single or multiple DNA

strands, is an excellent method for monitoring the biological processes associated with DNA damage.⁸ Although human studies of DNA damage have largely used lymphocytes, these cells are difficult to harvest, and the process is invasive and may cause discomfort to patients. Accordingly, researchers began to study buccal mucosa cells, which can be collected using minimally invasive procedures,⁹ and many studies have described the successful use of these cells in comet assays.^{7,9,10} In this study, therefore, the comet assay was implemented to detect DNA damage in human buccal mucosa cells exposed to radiation from panoramic radiography.

METHODS

Patient recruitment and cell sample collection

A total of 20 participants who underwent dental panoramic radiography for diagnosis and treatment at the Prof. Soedomo Dental Hospital Faculty of Dentistry, Universitas Gadjah Mada, Indonesia, were recruited. All participants were required to meet the following inclusion criteria: (1) No radiographic exposure for at least 2 weeks prior to the study; (2) good apparent health, with no smoking or alcohol consumption habit; (3) no lesions in the cheek mucosa; (4) no use of mouthwash. All participants signed informed consent forms, and the experimental protocol was approved by the Research Ethics Committee of the Faculty of Dentistry, Universitas Gadjah Mada, Indonesia (00679/KKEP/FKG-UGM/EC/2016).

Each participant was exposed to radiation via panoramic radiography performed using a Yoshida Panoura Deluxe system (Tokyo, Japan) with the following exposure parameters: 90 kVp, 8–10 mA, and 20 s. Subsequently, buccal mucosa cells were collected from the participants by gently scraping the inside cheek of the mouth with a cytobrush after rinsing the mouth with distilled water. The cytobrush was then agitated in a vortex for 30 s in a tube containing phosphate-buffered saline (PBS). The resulting buccal cell suspension was centrifuged at 2500 rpm and 4°C for 10 min, after which the supernatant was removed and the cell pellet was suspended in 30 µL of cold PBS. The participants were divided into two groups. Buccal mucosal samples were collected from both groups prior to radiation exposure and at 30 min or 24 h after radiation exposure in groups 1 and 2, respectively.

Comet assay

The comet assay was performed using an Oxiselect Comet Assay Kit (STA-351; Cell Biolabs, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, one volume of buccal cell suspension was added to 10 volumes of comet agarose and immediately transferred to the Oxiselect Comet Slide, which was placed in an aluminum foil-lined container at 4°C for

15 min. Subsequently, each slide was soaked in cell lysis buffer at 4°C for 60 min, after which the lysis buffer was replaced with a cold alkaline solution for 30 min. All slides were then subjected to electrophoresis in a horizontal chamber containing an alkaline electrophoresis solution at 18 V and 300 mA for 20 min, transferred to a clean container containing cold water, and washed three times. Following a final wash with cold 70% ethanol, the slides were dried completely in clean containers. Vista Green DNA dye (100 µL) was added to each fully dried slide and allowed to stand for 15 min at room temperature. Finally, the slides were observed using a fluorescence microscope (Leica GmbH, Berlin, Germany) connected to a camera and a computer. Cellular DNA damage was determined by measuring the percentage of tail DNA (tail DNA%) in 10–50 cells with OpenComet software, version 1.3 (www.cometbio.org), which analyzes comet assay images using an automated algorithm.¹¹

Data analysis

For each sample, the comet assay parameters were determined by measuring the percentage of tail DNA in at least 10 cells. The Shapiro–Wilk test was used to confirm the normal distribution of data, and the paired *t*-test was used to compare differences in samples collected before and after radiation exposure. STATA software version 13.1 (Stata Corp., College Station, TX, USA) was used to conduct the statistical analysis. A *p*-value of <0.05 with a 95% confidence interval was considered statistically significant.

RESULTS

Because even low-dose X-ray radiation has been shown to induce DNA damage, this study evaluated whether exposure to panoramic radiography could break DNA strands in the buccal mucosa cells collected from the study participants. In the cell, damaged DNA is distinct from intact DNA, and the former exhibits a comet tail-like appearance under a fluorescence microscope. Figure 1A shows representative photomicrographs of comets in buccal mucosa cells collected from a patient before and after exposure to X-ray radiation. The intact cells appear as spherical, homogeneously intense areas of fluorescence. By contrast, cells with DNA damage exhibit homogeneous fluorescence intensity in the comet head, surrounded by a sparsely fluorescent tail.

OpenComet software was used to detect the comet shapes based on parameters such as the convexity and head displacement ratios and thus separate valid and invalid comets; specifically, comets with a convex shape but irregular ratio were flagged as outliers. As shown in Figure 1A and B, buccal mucosa cells exhibit a low level of comet formation before radiation exposure, and an increase in this parameter can be observed at 30 min after radiation exposure. As shown in Figure

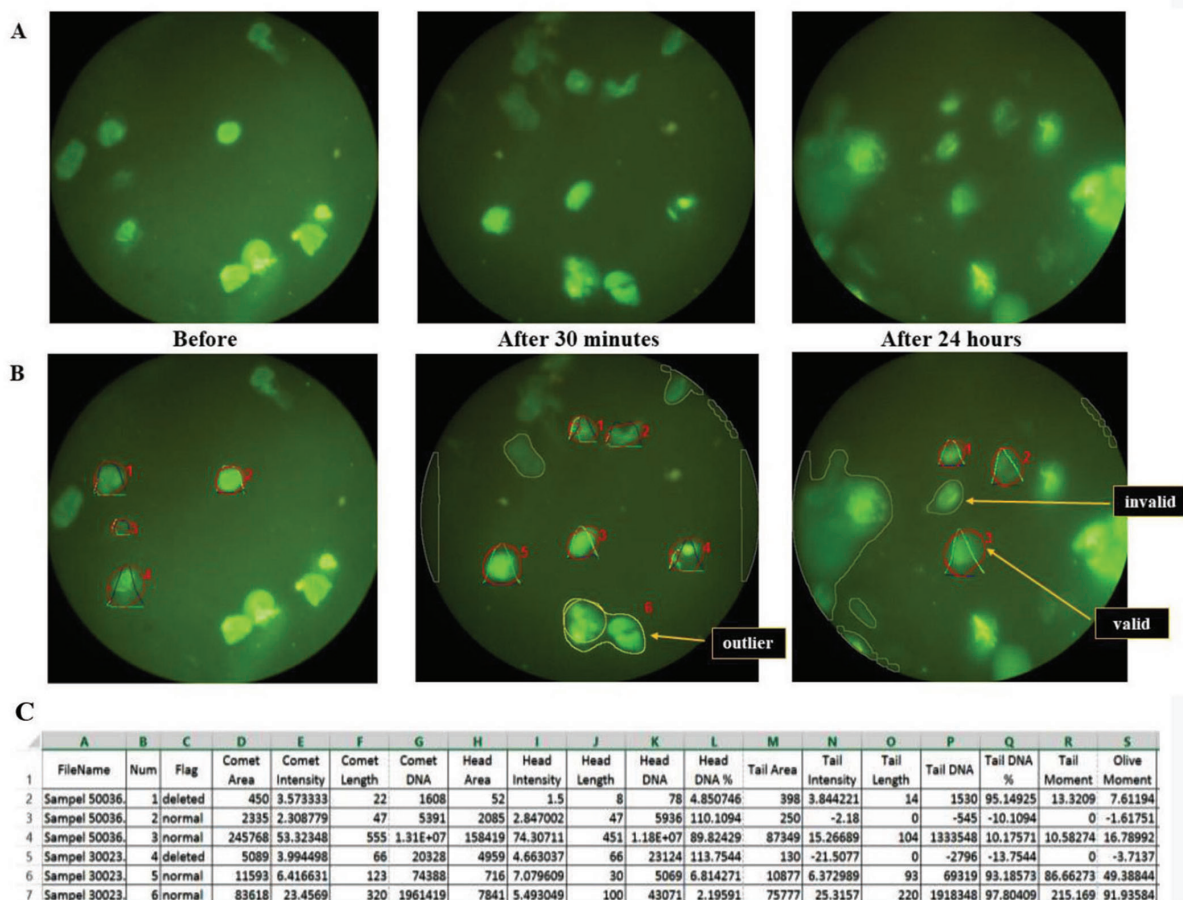


Figure 1. Detection of comet tails in buccal mucosal cells. (A) Representative fluorescence microscopy images (100× magnification). (B) OpenComet output image depicting valid (red outline), invalid (gray outline), and outlier (yellow outline) comets with profile plots and identification numbers. (C) Spreadsheet of the measurement results from OpenComet.

IC, the comet measurements and statistics used to estimate DNA damage in the cells were exported as a spreadsheet. From the several parameters reported by the software, tail DNA was selected because it covers the widest range of damage and exhibits a linear relationship with DNA double-strand breaks (DSBs)¹². Outlier comets were deleted from the statistical analysis to decrease the number of false positives, and therefore only 10–25 cells per sample were calculated. Buccal cells collected at 30 min after radiation exposure exhibited a statistically significant increase in the tail DNA% ($p < 0.05$; Figure 2A); by contrast, no significant difference was observed between samples collected before and at 24 h after exposure (Figure 2B). In other words, DNA damage increased in the buccal cells at 30 min after radiation exposure but had reverted to the pre-exposure state after 24 h.

DISCUSSION

This study aimed to evaluate X-ray-induced DNA damage in the buccal mucosa cells of patients

undergoing panoramic radiography using a comet assay that could detect ionizing radiation-induced damage such as DSBs. Although the simple, versatile, and sensitive comet assay is widely used to monitor the extent of DNA damage in human cells,^{8,10,11} to the best of the author’s knowledge, this is the first study to use the comet assay to observe DNA damage in human buccal mucosa cells subjected to radiation exposure from panoramic radiography.

The comet assay relies upon the fact that DNA molecules are negatively charged. A large, intact DNA molecule slowly migrates during electrophoresis, while smaller DNA fragments migrate more rapidly toward the anode to form a typical comet with a head comprising intact, undamaged DNA and a tail comprising damaged fragments.^{8,13} As shown in Figures 1 and 2, the buccal mucosal cells exhibited a background level of DSB and low tail DNA% before radiation exposure. This finding may be attributable to the low sample viability (~12.49%, unpublished data), consistent with a previous report indicating the low viability (12%) of buccal samples containing

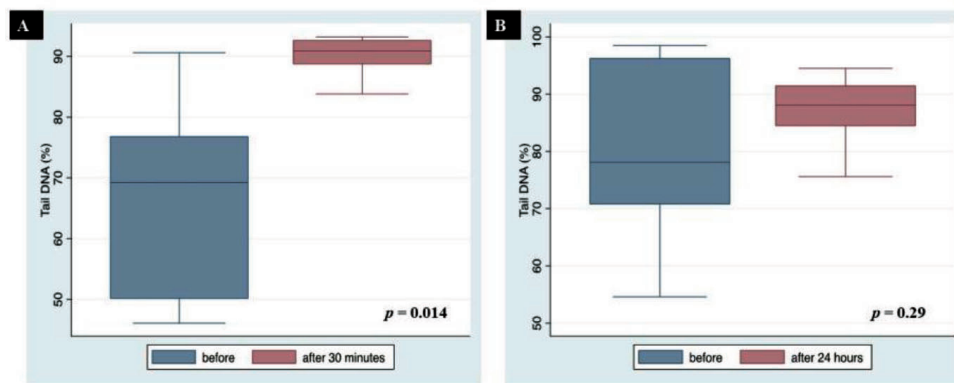


Figure 2. Box plot corresponding to changes in the percentages of tail DNA (%) in buccal mucosa cells from before to 30 min (A) or 24 h (B) after exposure to radiation. A p -value of <0.05 was considered significant.

epithelial cells.¹⁰ Dead or dying cells exhibit extensive fragmentation and appear as irregularly shaped areas of fluorescence intensity on photomicrographs of comet assays. Therefore, it is important to use viable cells in comet assays. Moreover, OpenComet software detected irregularly shaped DNA in the comet heads, which appeared as clouds without visible tails, as outliers or invalid comets.¹⁰ Large numbers of these DNA clouds were detected in most comet studies of buccal samples.^{10,14,15} Accordingly, in the present study, these outliers or invalid comets were excluded from the quantitative analysis to avoid overestimating DNA damage.

The extent of DNA damage is related to the amount of DNA in the tail,¹¹ although the optimal parameter for measuring DNA damage remains controversial. Three measures of DNA migration are commonly used: the tail length; olive tail moment (OTM), calculated as a product of the tail DNA% and tail length; and tail DNA%.¹² In this study, all comet assay parameters, including the tail area, tail DNA, tail DNA%, tail length, comet length, and OTM, were analyzed using OpenComet analysis software. Although OTM appears to be the most statistically significant measurement, inter-laboratory results are difficult to compare because a standard unit has not been set. Still, the tail length, which is used to calculate the OTM, is considered unsatisfactory because it increases only during tail production; increases in tail intensity without corresponding increases in length would not be counted. Moreover, cells from different tissues or different species can differ substantially in tail length. By contrast, the tail DNA% is insensitive to this effect^{8,12} and is thus preferred.

In this study, the tail DNA% was analyzed in buccal mucosa cells collected from patients before and after exposure radiation, and a statistically significant increase ($p < 0.05$) was observed in this parameter

at 30 min after exposure, but not at 24 h (Figure 2). In other words, exposure to X-rays induces DSBs in buccal mucosa cells shortly after exposure, although this effect is reversed after 24 h. This result could theoretically be attributed to cellular mechanisms that carefully maintain the repair of DNA breaks. Once DNA damage occurs, the Mre11-Rad50-Nbs1 (MRN) complex is activated and subsequently recruits a series of transducer (ATM, ATR, and 53BP1) and effector proteins to the sites of damage.¹⁶ The DSB breaks are then repaired by one of two known repair mechanisms, non-homologous end-joining and homologous recombination (HR),¹⁷ although the choice of pathway is not fully understood. Although the exact timing of initial DNA repair remains unclear, previous studies have attempted to detect this event. For example, a previous study of human blood samples observed greater than twofold changes in the expression of genes involved in specific DNA repair functions (XPC, DDB2, LIG1, POLH, and RAD51) at 24 h after exposure to 2 Gy of X-rays.¹⁸ Furthermore, two DNA damage response molecules, γ -H2AX and pChk2, were found to be expressed at approximately 0.1–48 and 0.25–32 h, respectively, after exposure to ionizing radiation from intraoral dental radiographs.¹⁹ Therefore, it would be reasonable not to detect DSBs at 24 h after exposure.

The above time course data suggest that further research is needed to reveal the involved molecules. A follow-up study regarding the associations of genes involved in DNA damage and repair and the corresponding time windows would help understand the mechanism underlying the effects of low-dose radiation in buccal mucosal cells. One additional limitation of the present study is the absence of a comparative analysis with measurements obtained using other software, as both commercial and free software packages have known advantages and limitations. Moreover, this study did not analyze all output parameters generated by

OpenComet software. Therefore, a future study should use multiple software packages and parameter analyses to yield more accurate interpretations of the results.

CONCLUSION

In conclusion, the results of the present study suggest that DSBs can be induced in buccal mucosal cells at 30 min after exposure to radiation from panoramic X-rays, thus underscoring the clinical importance of the three principles of radiation protection: justification, optimization, and dose limitation. Given the causative role attributed to DNA damage in the processes of cell lethality and mutation leading to carcinogenesis, these data may be relevant to human health risk assessments. Protective measurements should be considered for patients undergoing panoramic radiography as the study results suggest that even low-dose radiation can induce DNA damage.

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CONFLICT OF INTEREST

The authors declare that there were no conflicts of interest related to this study.

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