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Viability and DNA Damage of Buccal Mucosa Cells in Patients Exposed to Panoramic X-ray

Ryna Dwi Yanuaryska*, Afit Aditya Atmoko, Isti Rahayu Suryani, Rurie Ratna Shantiningsih

Department of Dentomaxillofacial Radiology, Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia

*Corresponding author: ryanuaryska@ugm.ac.id

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ABSTRACT

Panoramic X-ray is well known to cause DNA damage and induces cellular death. The aim of the present study was to evaluate the cytotoxicity of radiation exposure from panoramic radiography on human buccal mucosa cells by assessing the cell viability using the simple-trypan blue exclusion test. The genotoxicity effect was evaluated by assessing comet assay score. This research included a total of 20 healthy patients who had panoramic radiography for a routine dental examination. Buccal mucosa cells were collected from all participants before X-ray exposure and at 30 min or 24 h after exposure in Groups 1 and 2, respectively, and subjected to a comet assay and trypan blue exclusion test to assess cell viability and DNA damage. Cell viability was calculated as the ratio of live (translucent) to total counted cells. Comet assay output images were analysed using OpenComet software and a visual score by measuring the percentages of tail DNA and summing the visual score, respectively. A statistically significant ($p < 0.05$) reduce in cell viability was observed at 30 min after exposure, furthermore there is no more reduction after 24 h. Both comet assay measurements showed a significant ($p < 0.05$) increase in the percentage of tail DNA and visual score at 30 min after exposure, then tend to decrease after 24 h of exposure, although it was not significant ($p > 0.05$). The results showed that panoramic radiography interfered cell viability and induced DNA damage in buccal mucosa cells within 30 min after exposure, but these effects were ceased after 24 h.

Keywords: *Buccal mucosa; cell viability; comet assay; DNA damage; panoramic radiography*

INTRODUCTION

Panoramic radiograph provides an overall facial image including both the maxillary and the mandibular dental arches and their supporting structures. The dose used in panoramic radiography is relatively low, about 2.7 to 38 μSV. However, this dose is below the radiation exposure dose limit as required by the International Commission on Radiological Protection (ICRP) of 0.3 mSv. Nonetheless, low doses of X-ray radiation have an adverse effect and may produce biological changes in the living tissues. ICRP for radiation safety makes conservative assumption that the biological damage caused by ionising radiation is related to radiation exposure, regardless of the size of the dose. Therefore, all doses of radiation are considered harmful (Lee *et al.*, 2014).

Research on the effects of panoramic radiography has been widely done (Cerqueira *et al.*, 2008; Angelieri *et al.*, 2010; Ribeiro,

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2012) and showed that panoramic radiography causes chromosomal damage, nuclear changes and cell death. In agreement with our previous research, panoramic X-ray radiation induced double-strand breaks (DSBs) in buccal mucosa cells within 30 min after exposure (Yanuaryska, 2018). Beside DNA damage, other interesting discoveries by Cao *et al.* (2015) in the experiment using Saccharomyces cerevisiae showed that changes in cell membrane integrity and permeability were induced by X-ray irradiation.

Saccharomyces cerevisiae (*S. cerevisiae*) is one of the most well-known eukaryotic models as it shares a complex intracellular organisation with the higher eukaryotes. This gives advantages in the study of cellular processes such as basic biology of the cell cycle, membrane trafficking, lipid metabolism and many other cellular systems (Feyder *et al.*, 2015). X-ray irradiation induces significant damage to cell membranes of *S. cerevisiae*, in conjunction with a decrease in cell viability (Cao *et al.*, 2015). To the best of our knowledge, the effects of X-ray irradiation from panoramic radiography on cell viability of human buccal cells have not yet been investigated.

Damage to cell membranes can be assessed by measuring the protein permeability, diffusion rates of nucleic acids, and staining with specific fluorescent dyes (Cao *et al.*, 2015). At the same time, these methods are typically laborious and time-consuming. Therefore, the dye exclusion test is used to determine the number of viable cells present in buccal mucosa cells from patients exposed to X-ray via panoramic radiography. It is based on the principle that living cells have intact cell membranes which drive out certain dyes, such as trypan blue, while dead cells do not have it (Strober, 2015).

MATERIALS AND METHODS

Subjects

The current study was conducted after obtaining approval from The Medical and Health Research Ethics Committee, Faculty of Medicine, Universitas Gadjah Mada (UGM) and Dr. Sardjito General Hospital (KE/FK/0649/EC/2018). Furthermore, signed informed consent from each participant from a total of 20 participants who underwent panoramic radiography for diagnosis and treatment in Professor Soedomo Dental Hospital, UGM, Indonesia was also taken. All participants were required to meet the following inclusion criteria: (1) Good apparent health, with no smoking or alcohol consumption habit; (2) Age between 18 and 25 years; (3) No lesions in the cheek mucosa; (4) No use of mouthwash; and the exclusion criteria: (1) The presence of systemic diseases or being differently abled; (2) Radiographic exposure for at least two weeks prior to the study. Each participant was exposed to radiation via conventional panoramic radiography which was performed using Yoshida Panoura Deluxe system (The Yoshida Dental MFG. Co., Ltd., Tokyo, Japan) with following exposure parameters: 90 kVp, 8–10 mA, 20 s.

The participants were divided into two groups. Buccal mucosa 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. After gently rinsing the mouth with distilled water, cells were obtained by scraping the inside cheek of the mouth with a cytobrush. The cytobrush was then agitated in a vortex for 30 sec in a tube containing phosphate-buffered saline (PBS). The resulting buccal cell suspension was centrifuged at 2,500 rpm and 4°C for 10 min, after which the supernatant was removed, and the cell pellet was suspended in 40 µL of cold PBS.

Trypan Blue Exclusion Test

The cell suspension (10 μL) was mixed by trypan blue 0.4% (Sigma Aldrich, Darmstadt, Germany) and the cells were counted using an Improved Neubauer hemocytometer (Paul Marienfeld GmbH & Co. KG, Lauda-Konigshofen, Germany). The numbers of live (translucent) and dead (stained) cells were determined by a bright field microscope (200×). Cell viability was calculated as the ratio of live to total counted cells.

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 instruction. The cell suspension (30 μL) was added to comet agarose (ratio v/v 1:10) then transferred onto the Oxiselect Comet Slide at once and placed on aluminium foil coated container at 4°C for 15 min. The slide was then soaked in a cell lysis buffer at 4°C for 60 min. Lysis buffer was removed and replaced with a cold alkaline solution at 4°C for 30 min. The slides were then placed in horizontal chamber electrophoresis containing alkaline electrophoresis solution and run at 18 V, 300 mA for 20 min. The slides were moved into a clean container containing cold water and washed 3×. The final washing was done with cold ethanol 70% then placed on dry and clean containers to allow drying completely. Once the agarose and slide were completely dry, 100 μL of Vista Green DNA dye was added to the slide and allowed to stand for 15 min at room temperature. The slide was observed under fluorescence microscopy (Leica, Germany) connected to the camera and computer. DNA damage was determined by measuring the tail DNA % of at least 50 to 100 cells per sample with OpenComet software 1.3 (Gyori *et al.*, 2014) and was also compared with visual scores (Collins *et al.*, 2008).

Data Analysis

Repeated ANOVA was performed to compare if there were differences in tail DNA % and cell viability between the groups: before, after 30 min, after 24 h. To assess the differences in visual scores of DNA damage between the groups, we used the non-parametric test, Friedman test. Statistical analysis was performed in IBM SPSS Statistics for Windows version 22.0 and a *p*-value of less than 0.05 was considered statistically significant with a 95% confidence interval.

RESULTS

Panoramic X-ray is known to have cytotoxic and genotoxic effects on the cell. To assess the cytotoxic effect, we measured cell viability by using trypan blue exclusion in human buccal mucosa cells at before, 30 min, and 24 h after X-ray exposure. Viable cells marked as translucent cells, meanwhile the nonviable cells appear blue as a consequence of taking up trypan blue (Fig. 1[a]). Panoramic X-ray significantly reduced cell viability (Fig. 1[b]) in 30 min after exposure which remained after 24 h.

Genotoxic effect of panoramic X-ray was proved by measuring DNA damage in the comet assay image. Damaged cellular DNA was separated from intact DNA, yielding a typical comet shape under a microscope (Fig. 2[a]). As demonstrated in Fig. 2(b), panoramic X-ray significantly increase the percentage of tail DNA to 16% at 30 min after exposure, then slightly decrease at 3% ($p > 0.05$) after 24 h of exposure. The percentage of tail DNA is a commonly used parameter to analyse comet assay results. Additionally, the different approaches were used in this study, i.e., visual scoring, to confirm the data obtained from OpenComet software. In visual scoring, comets were classified into five categories: 0 representing undamaged cells (no or barely detectable tails) and 1 to 4 representing damaged

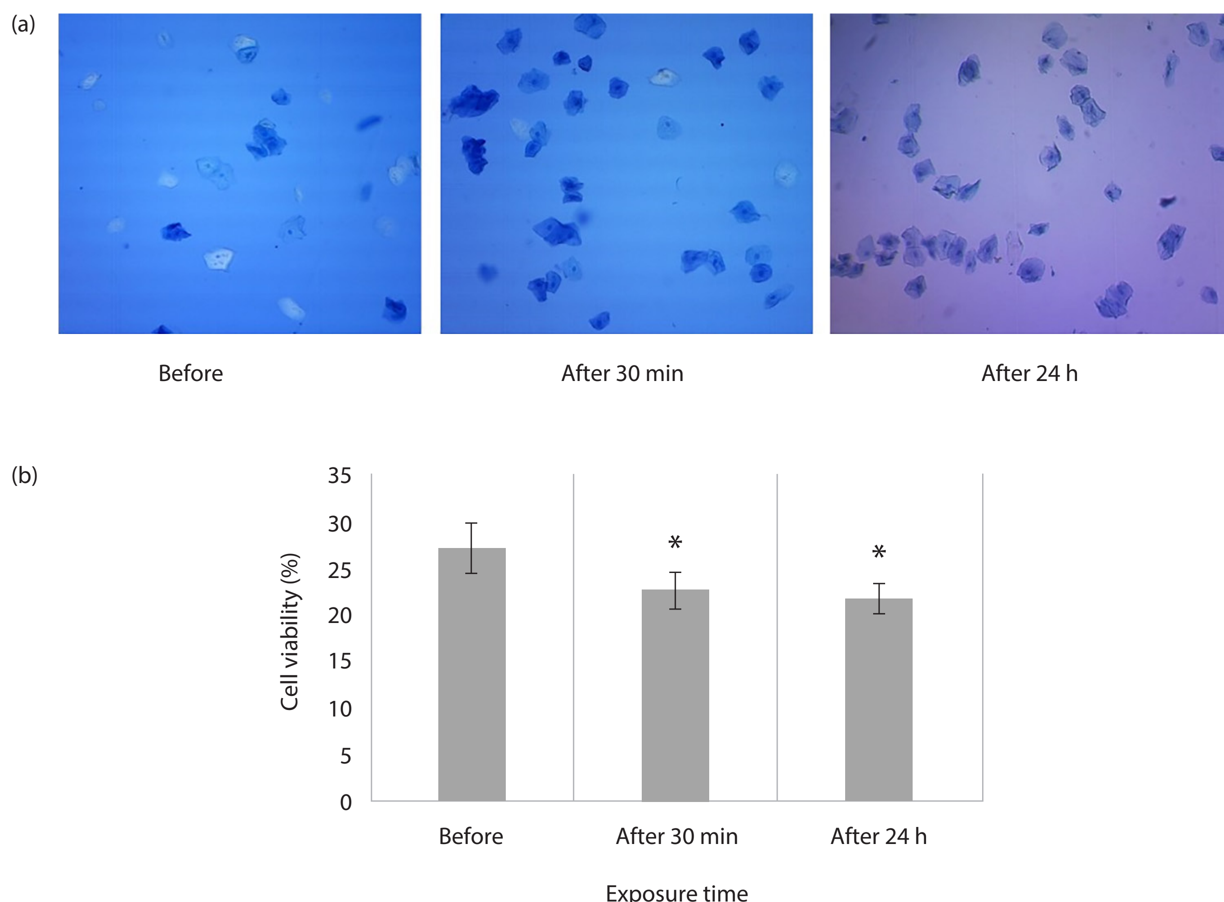

Fig. 1 The viability of human buccal mucosa cells. (a) Representative photomicrograph of cells that were stained with trypan blue (100× magnification). Note the increase of dead (stained) cells after 30 min and 24 h compared with before exposure; (b) Quantification of the cell viability and there was significant difference (* $p < 0.05$) compared with before exposure.

cells by increasing relative tail intensities (Collins *et al*., 2008; Ganapathy *et al*., 2016). The samples obtained before exposure consisted of undetectable and barely detectable comet shape, on the other hand, after 30 min and 24 h of exposure, mild and moderate comet shape were superiority found. Extensive comet shape was rarely found in those samples. Summing the scores (0 to 4) of 60 cells per sample gave an overall score of between 0 to 240 (no units), which was used for statistical analysis. Fig. 2(c) illustrates the evidence of visual scoring exhibit a trend in DNA damage in coherence with the tail DNA percentage. Visual score rose around 24% at 30 min after exposure, then declined by about 6% ($p > 0.05$) after 24 h of exposure.

DISCUSSION

This study aimed to evaluate the cytotoxic and genotoxic effect of panoramic X-ray in human buccal mucosa cells by measuring the cell viability and DNA damage. Researchers had worked on the micronucleus test and nuclear alteration such as karyorrhexis, pyknosis and karyolysis (Angelieri *et al*., 2007; Ribeiro *et al*., 2011; Preethi *et al*., 2016). In this present study, the different approaches were used, which no such study had reported previously. The simple trypan blue exclusion and comet assay were used to evaluate the effects. There was only one literature found about X-ray effect on membrane permeability and integrity. The research was conducted on *S. cerevisiae*

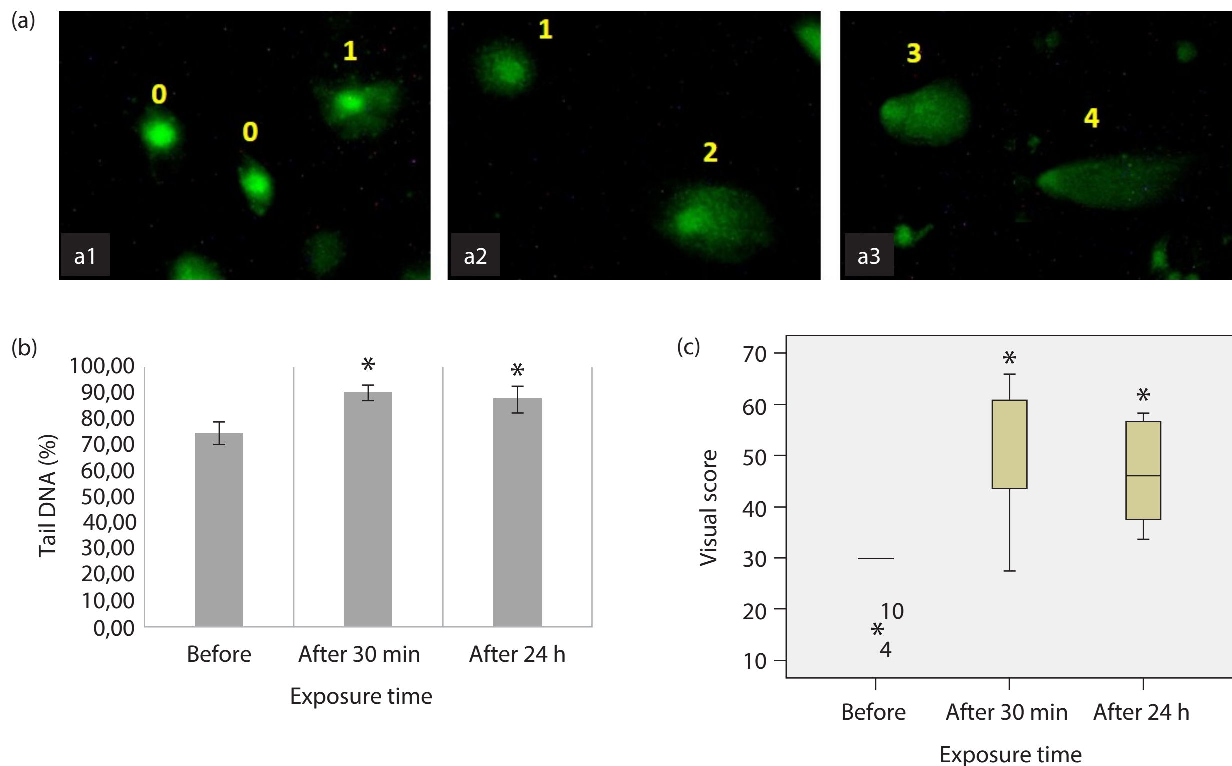

Fig. 2 Detection of DNA damage by comet assay. (a) Representative fluorescence microscopy images (100× magnification) in buccal mucosa cells from (a1) before- and (a2) after 30 min or (a3) 24 h of exposure. Comet shape is undetectable in 0, barely detectable in 1, mild detected in 2, moderate in 3, and extensive in 4; 100× magnification; (b) Quantification of tail DNA (%) in comet assay image measured by OpenComet software; (c) Quantification of DNA damage in comet assay image by the visual score and there was significant difference (* $p < 0.05$) compared with before exposure.

which served as a eukaryotic model in radiation biology studies. X-ray irradiation increases permeabilised cell number and decrease overall esterase activity thus resulting increase of non-viable cell numbers (Cao *et al.*, 2015). Those results may explain the decrease of cell viability in buccal mucosa cells at 30 min after panoramic X-ray exposure. Ionising radiation leads to membrane depolarisation, i.e., displacement of the negative charge from intracellular outward cells so that the charge inside the cell becomes less negative (Somosy, 2000).

Cell permeabilisation and loss of metabolic activity as a result of X-ray irradiation may be an irreversible phenomenon (Cao *et al.*, 2015). The number of negatively charged on the surfaces of human fibroblasts reduced significantly within 1 min after X-irradiation, in the dose range from 0.25 to 2.5 Gy, then recovered within 1 h. The reversibility of radiation-induced changes depends on the cell type and the source of radiation (Somosy, 2000). Therefore, it would be reasonable that there was no significant difference ($p > 0.05$) on cell viability at 30 min and 24 h after exposure. This may be due to an adaptive response of buccal mucosa cells induced by exposure to X-ray radiation. Adaptive responses such as protection against reactive oxygen species, DNA repair and elimination of genomically damaged cells, are activated by low doses of ionising radiation that trigger repair and protective processes to prevent further damage (Desouky *et al.*, 2015). These phenomena coincide with DSBs as showed in the comet assay results.

Panoramic X-ray induced DNA damage within 30 min after exposure and slowly repaired during 24 h. This fact could theoretically be explained that cells have mechanisms in the body which maintain the repair of DNA breaks. DSBs are primarily repaired by one of two pathways, non-homologous end-joining (NHEJ) and homologous recombination (HR), although the choice of the pathway is not fully understood. However, heavy ion induced DSBs, which are repaired slowly, are more often repaired by HR, whereas X- or γ-ray induced breaks are repaired via NHEJ (Jeggo *et al.*, 2011). The exact timing of initial DNA repair remains unclear. However, two DNA damage response molecules, γ-H2AX, and pChk2 were detected in time range around 0.1 h to 48 h and 0.25 h to 32 h, respectively, after ionising radiation exposure from intraoral dental radiographs (Yoon *et al.*, 2009). Therefore, it could be assumed that there was a repair process of DSBs induced by panoramic X-ray during 24 h after exposure.

CONCLUSION

The present study gave a new insight regarding the panoramic X-ray effect on cell viability and DNA damage. The results strongly suggested that panoramic X-ray discharged cell viability and induced DSBs within 30 min after exposure. Even though at low doses, panoramic X-ray induce cytotoxicity and genotoxicity in human buccal mucosa cells. Radiation protection in the patient undergoing panoramic radiography should be considered as protecting the human health per se. However, further analyses are required to investigate the associations of cell viability and DNA damage therewith the involved molecules.

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