Original Article

Differential Proliferative Responses to Low-Dose Irradiation between Human Submandibular and Parotid Salivary Ductal Cell Lines

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Objective: Salivary glands are frequently exposed to X-radiation during dental radiography. The present study aimed to investigate the proliferative and apoptotic effects of low-dose irradiation on human salivary cell lines in vitro.

Materials and Methods: Two distinct ductal cell lines, HSG and HSY, were first characterized by expressions of carcinoembryonic antigen (CEA) and lactoferrin (LF), compared to those in human oral keratinocytes (HOKs), and then examined for their doubling times. They were exposed to periapical radiography for 5, 10, or 20 times, and incubated further for 1, 3, or 5 cycles of their cell division. Cell proliferation was examined by a BrdU assay and immunoblot analysis of Ki-67 expression. Cell apoptosis was determined by the presence of human active caspase 3.

Results: The mean degrees of CEA and LF expressions were significantly higher in HSG and HSY than in HOKs ($p<0.01$). The doubling time of HSG was significantly shorter than that of HSY ($p=0.009$). Upon repeated exposures to dental X-ray, the proliferation of HSG was significantly increased at the first cycle, whereas that of HSY was significantly decreased ($p<0.05$). At the third cycle, Ki-67 expression was significantly increased in HSG, while it was significantly decreased in HSY ($p=0.037$). However, the proliferative effect was temporarily presented in both cells. No active caspase 3 was detected upon exposure to any doses or in any cycles.

Conclusion: The two ductal cell lines demonstrated adaptive response to low-dose irradiation by either increased or decreased cell proliferation, but no apoptosis was found in both cell lines.

Keywords: Bromodeoxyuridine assay; Cell apoptosis; Cell proliferation; Dental X-ray; Salivary ductal cells

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X-radiation is an ionizing electromagnetic wave with high energy. In diagnostic imaging, not all X-ray reaches to an X-ray film or a digital receptor. Instead, scattered radiation absorbed by the patient's tissues can cause chemical changes, followed by biological damages within the cells[1]. According to the 2012 report of the United Nations Scientific Committee on the Effects of Atomic Radiation, low doses of irradiation are referred to the amounts of radiation below 100 mGy[2]. In dentistry, tissues and organs are designated as "critical" because they are more vulnerable and frequently exposed to radiation than others during imaging procedures. A critical organ is an organ that, if damaged, would diminish the quality of life. The critical organs, exposed to X-ray during various dental imaging procedures in the head and neck region, include thyroid glands, bone marrow, skin, lens of the eye, brain, and salivary glands[3]. A salivary gland consists of multiple secretory acini

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connected to the oral cavity by their ductal system[4]. Small intercalated ducts, directly adjoined to the secretory end pieces, are found within the lobar structure of salivary gland. It is believed that a major function of ductal cells within the intercalated duct is to provide a reservoir for progenitor cells, capable of regeneration into specialized components of the gland[5,6].

Previous studies have demonstrated collateral damages of high-dose irradiation, of more than 1 Gy, used to treat head and neck cancer on salivary glands[7]. However, the findings from these studies are not applicable for the effects of low-dose dental X-ray on salivary glands or cells. In general, cell-based studies using various cell types other than the salivary cells indicate that there are no long-term cytogenetic changes in association with dental X-ray exposure. However, localized cytotoxic responses are still detected in these cells[8-12]. Consequently, there has not yet been any study to conclude whether low-dose dental X-ray causes any adverse effects on human salivary cells.

In the present study, two distinct salivary ductal cell lines, HSG and HSY, were used as a valuable tool to uncover regulatory events and cellular responses of salivary cells to dental X-ray. The HSG was originally isolated from the submandibular salivary gland removed during radical neck dissection and radiotherapy for squamous cell carcinoma at the floor of the mouth[13], while the HSY was derived from adenocarcinoma of the parotid gland[14]. Therefore, the objective of the present study was to determine cell reactions, including both proliferative and apoptotic effects, upon low-dose X-ray exposures in cultured HSG and HSY.

Materials and Methods

Cell culture

HSG and HSY were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco) in a 5% CO_2 humidified incubator. Human oral keratinocytes (HOKs) from five healthy volunteers isolated from non-inflamed gingival biopsies overlying bony impaction of third molars[15] were cultured in keratinocyte growth medium (PromoCell, Heidelberg, Germany). The rationale for use of HOKs as a cell line control was because both salivary ductal epithelial cells and HOKs are derived from the same oral ectodermal lineage, lining the primitive oral cavity of human embryos; however, the invaginated salivary cells are later differentiated to become specialized ductal or acinar cells that express specific salivary markers[16].

Characterization of HSG and HSY

HSG and HSY were first characterized for expressions of carcinoembryonic antigen (CEA) and lactoferrin (LF) by immunoblotting. The protein contents were determined by a protein quantification BCA assay kit (Pierce, Rockford, IL, USA) before a 10-µg aliquot of protein from each sample was resolved on SDS-PAGE and transferred to nitrocellulose membranes. The membrane was incubated with the anti-CEA (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), the anti-LF (1:2,000; Santa Cruz Biotechnology), or the anti-β-actin antibody (1:1,000; Santa Cruz Biotechnology), followed by reaction with the HRP-conjugated secondary antibody (1:2,000; DAKO, Glostrup, Denmark). The membrane was reacted with the LumiGLO Reserve® chemiluminescence detection reagent (KPL, Gaithersburg, MD, USA). Protein bands were visualized under the ChemiDoc™ XRS gel documentation system (Bio-Rad Laboratories, Hercules, CA, USA) and captured by an attached CCD camera. The intensities of CEA and LF bands in each sample were quantified using Image J software (NIH, Bethesda, MD, USA), and normalized by that of β-actin band.

For immunofluorescence, the cells, grown in glass slide chambers (Lab-Tek®, Sigma-Aldrich, St. Louis, MO, USA), were reacted with the anti-CEA (1:100) or the anti-LF (1:100) antibody for one hour. After washing, the NorthernLights™ 557-donkey IgG (1:500; R&D Systems, Minneapolis, MN, USA) and the Alexa Fluor™ 488-phalloidin (1:500; ThermoFisher Scientific, Rochester, NY, USA) were added, followed by addition of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Biotium Inc., Hayward, CA, USA) for 10 minutes. The glass slides were mounted with fluorescent mounting medium (DAKO), and the signals were visualized and captured under a fluorescence microscope (Axio Imager Fluorescence Microscope, Carl Zeiss, Jena, Germany).

Assessment of population doubling time (PDT)

HSG and HSY, originally seeded in 6-well plates at 2×10^4 cells/ml, were detached from the plates on day 6. The PDT was calculated from the number of cells seeded on day 1 (N_{1d}), that on day 6 (N_{6d}), and the number of cultured days (ΔT) by an equation below:

$$PDT = \frac{\log (2) \times \Delta T}{\log (N_{6d}) - \log (N_{1d})}$$

Irradiation protocol

HSG and HSY, cultured in 96-well plates for cell proliferation or in 35-mm dishes for immunoblotting, were irradiated for 5, 10, or 20 times of repeated exposures to a standard dose of 60 kVp and 0.16 seconds each dose, of a digital periapical radiograph, using a dental X-ray machine generator (Heliodent Plus HF, Sirona, Germany). The amount of X-ray, measured by an X-ray test device (TNT 12000D®, Fluke Biomedical, Cleveland, OH, USA) for a single absorbed dose, was 1.1 mGy. To simulate multiple X-ray exposures of full-mouth radiography, with a maximum at 20 exposures as in clinical setting, a 60-second waiting time interval between each exposure was allowed.

Cell proliferation

After being irradiated, the cells were further cultured for their first, third, or fifth cycle of cell division, determined by PDT. The BrdU cell proliferation assay kit (Roche Diagnostics, Mannheim, Germany) was used to examine the effect of irradiation on cell proliferation, according to the manufacturer's instructions. Briefly, 10 µM BrdU was added into DMEM for four hours at 37°C, and the cells were later fixed with FixDenat solution for 30 minutes and incubated with 100 µl of the anti-BrdU-POD working solution for 90 minutes. After washing, the substrate solution was added and incubated for 30 minutes, and the reaction was stopped by 1 M H_2SO_4. The absorbance value was measured using a microplate reader (Sunrise™, Tecan, Männedorf, Switzerland). The percentage of cell proliferation in the experimental sample was derived by comparison with that of non-irradiated control at the same cycle of cell division, set to 100. Moreover, the effect of irradiation on cell proliferation was determined by Ki-67 expression. Extracted whole cell lysates were subjected to SDS-PAGE and immunoblotting. The membrane was reacted with the anti-Ki-67 (1:500; Santa Cruz Biotechnology) or the anti-β-actin antibody, followed by incubation with the HRP-conjugated secondary antibody. The band intensity of Ki-67, normalized by that of β-actin, in each sample was compared to that in the non-irradiated control at the same cycle of cell division, set to 1.

Cell apoptosis

After being irradiated, the cells were further cultured for their first, third, and fifth cycle of cell division. Cell apoptosis was determined by the presence of active caspase 3. Immunoblotting was conducted using the anti-caspase 3 antibody (1:2,500; Abcam, Cambridge, UK). As a positive control for cell apoptosis[17], HSG and HSY were treated with 0.1 M staurosporine (Sigma-Aldrich) or 0.1% (v/v) DMSO, as a vehicle control, for four hours before immunoblotting.

Statistical analysis

Differences in the mean degree of CEA or LF expression in HSG or HSY, compared to that in HOKs, and in the mean doubling time between HSG and HSY were tested by Student's t-test. Differences in the mean percentages of cell proliferation among different doses of X-irradiation, compared to that of the non-irradiated control within the same cycle of cell division, were determined by ANOVA, followed by Duncan test for multiple comparisons. Differences in Ki-67 expression between samples within the same cycle of cell division were determined by Mann-Whitney U test. The p-value of less than 0.05 was considered to be statistical significance.

Ethics approval and consent to participate

Use of primary human oral keratinocytes in the present study was approved by the Human Experimentation Committee, Faculty of Dentistry, Chiang Mai University (#32/2020).

Results

Characterizations of HSG and HSY

HSG and HSY were tested for expressions of CEA[18,19] and LF[19,20] by immunoblot and immunofluorescence analyses. Expressions of CEA and LF were detected in HSG and HSY compared to weak CEA and LF expressions in normal primary HOKs (Figure 1A). The mean degrees of CEA and LF expressions in HSG and HSY were significantly higher than those in HOKs ($p<0.01$) (Figure 1B, C). However, the degree of CEA or LF protein expression in HSG was not significantly different from that in HSY (Figure 1A-C). By immunofluorescence, both CEA and LF (red signals) were found in HSG and HSY. Consistent with weak expressions of CEA and LF, no red signal of either CEA or LF was detected in HOKs (Figure 1D). It was noted that a much larger size of HOKs than that of HSG or that of HSY.

Difference in PDT between HSG and HSY

Due to variations for the effects of low-dose

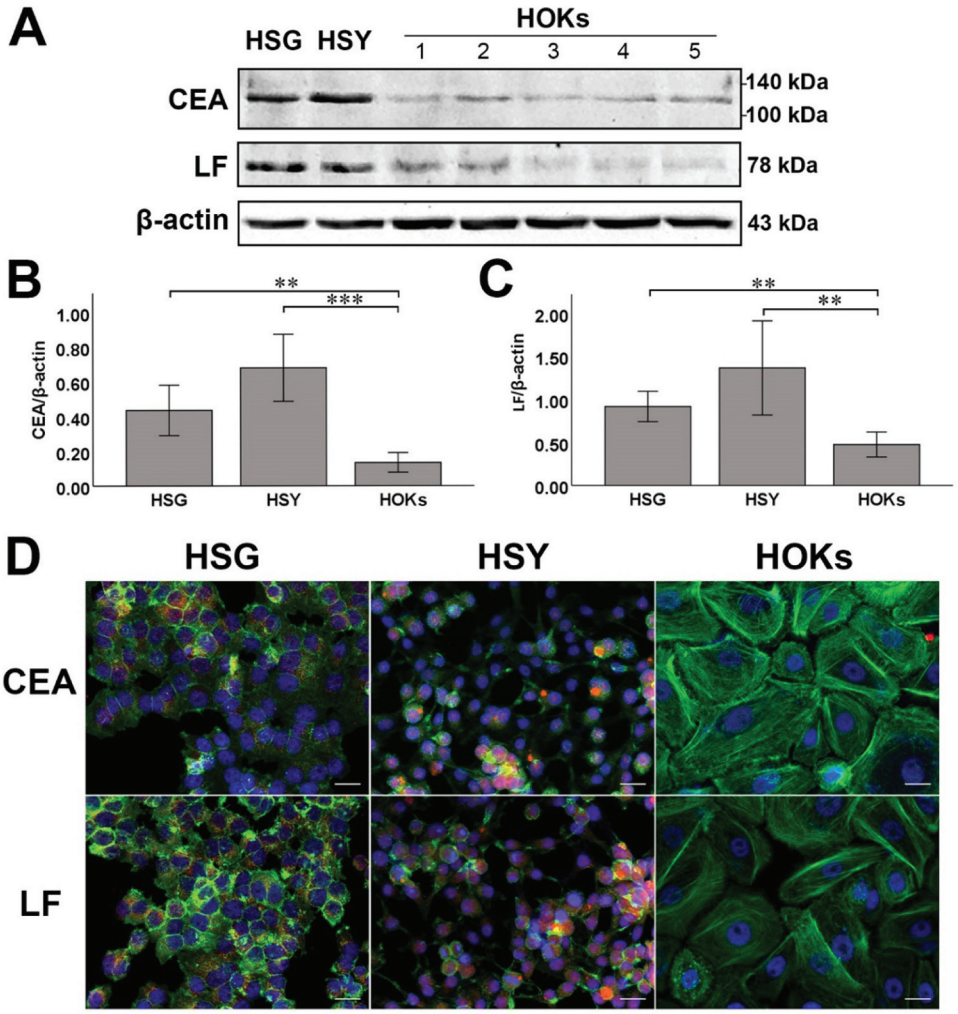
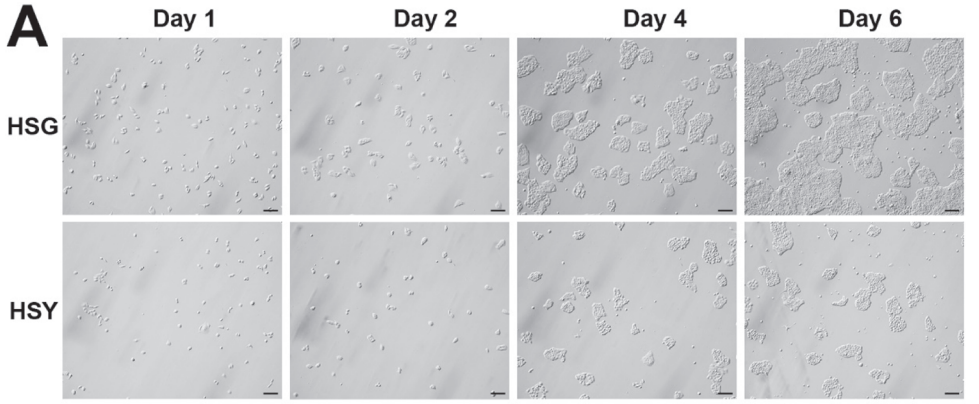


Figure 1. (A) Representative blots from five separate experiments (n=5) of carcinoembryonic antigen (CEA), lactoferrin (LF), and β-actin expressions in HSG, HSY, and human oral keratinocytes (HOKs) from five volunteers (1-5). The sizes of CEA and LF are as predicted. (B, C) The ratios of CEA and LF expressions against β-actin expression, respectively, in HSG, HSY, and HOKs. Error bars represent standard deviation; ** $p<0.01$, *** $p<0.001$. (D) Representative immunofluorescence of HSG, HSY, and HOKs, stained with the anti-CEA or the anti-LF antibody, followed by secondary antibody conjugated with NorthernLights™ 557 (red). The cell nuclei were indicated by DAPI staining (blue); the cytoplasm was visualized by staining with Alexa Fluor-488 conjugated phalloidin (green) for actin microfilament. Scale bars=20 μm.

irradiation in cells with different growth rates[1], the PDT of HSG and HSY were first assessed. Both HSG and HSY were first seeded at the same cell density on day 1; however, the colonies of HSG were found to be larger than those of HSY on days 4 and 6 (Figure 2A). By determination of PDT from three separate experiments, the mean PDT of HSG was 24.22±1.54 hours, while that of HSY was 30.25±1.55 hours (Figure 2B). The mean PDT of HSG was found to be significantly shorter than that of HSY ($p<0.01$) (Figure 2B). Therefore, in the subsequent irradiation experiments, additional culture times after irradiation for the first, third, and fifth cycles of cell division for HSG or HSY were at 24 or 30 hours, 72 or 90 hours, and 120 or 150 hours, respectively.

Differential proliferative responses to low-dose irradiation between HSG and HSY

At the first cycle of cell division, the mean percentage of cell proliferation rate, as assessed by the BrdU assay, in irradiated HSG repeatedly exposed to dental X-ray for 20 times was found to be significantly increased compared to that in the non-irradiated control ($p<0.05$) (Figure 3A). By contrast, the mean percentage in irradiated HSY for 10 or 20 times was found to be significantly decreased ($p<0.05$)



B

Cells	PDT (h)	Mean ± SD	P-value
HSG-1	24.27		
HSG-2	25.74	24.22 ± 1.54	
HSG-3	22.65		0.009
HSY-1	29.94		
HSY-2	28.89	30.25 ± 1.55	
HSY-3	31.93		

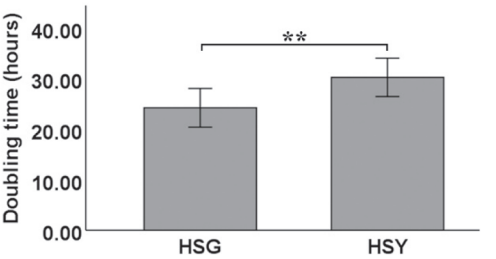


Figure 2. (A) Representative images from phase-contrast inverted microscopy of HSG and HSY cultured on days 1, 2, 4, and 6. (B, left) Data of population doubling time (PDT) in HSG and HSY from three separate experiments (n=3), expressed as a unit of hours (h). (B, right) A bar graph showing mean PDT ± standard deviation (error bars) of HSG and HSY; ** p<0.01. Scale bars=250 µm.

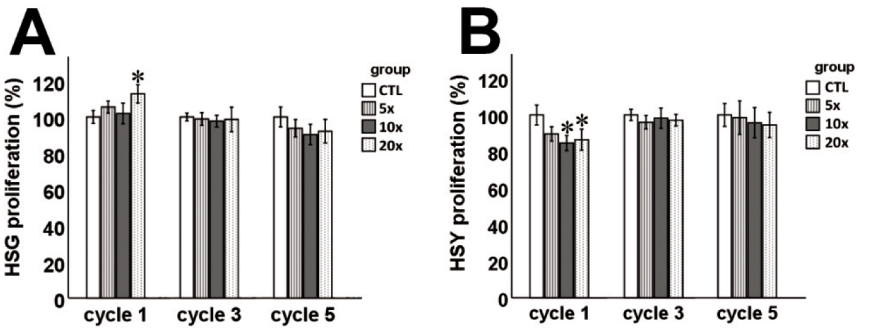


Figure 3. Bar graphs showing the percentage of cell proliferation, analyzed by a BrdU assay, at different cycles (1, 3, or 5) of cell division after HSG (A) and HSY (B) were repeatedly exposed to dental X-ray for 5, 10, or 20 times (x); CTL=non-irradiated cells; Error bars=standard deviation; n=4; * p<0.05.

(Figure 3B). However, the increased or decreased effect on cell proliferation was temporarily found in both cells. By immunoblotting, expression of Ki-67, a marker of mitotic (M) phase[21], was upregulated in HSG upon repeated exposures to dental X-ray for 5, 10, or 20 times at the third cycle of cell division (Figure 4A), whereas the Ki-67 expression was downregulated in HSY upon repeated exposures for 10 or 20 times (Figure 4B). By densitometric analysis, significant increases in the median Ki-67 expression were found in HSG exposed to dental X-ray for 5, 10, or 20 times at the third cycle of cell division (p=0.037) (Figure 4C), while significant decreases in the median Ki-67 expression were found in HSY exposed to dental X-ray for 10 or 20 times (p=0.037) (Figure 4D).

To determine cell apoptosis, immunoblotting for expression of the effector caspase, i.e., human caspase 3, and for the presence of its cleaved form of active caspase 3, one of the apoptotic markers, was conducted. It was demonstrated that no cleaved band of active caspase 3 was detected in HSG or HSY upon exposures to any doses of X-rays after observation at different cycles, as compared to the presence of

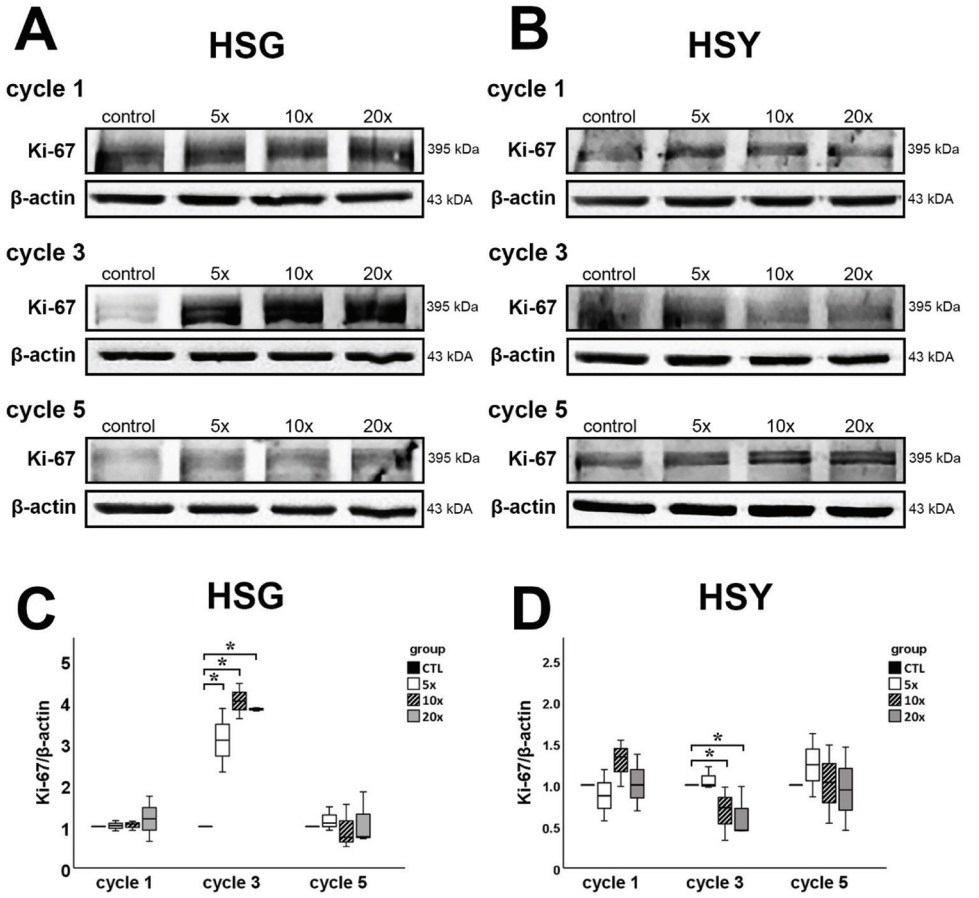


Figure 4. (A, B) Representative blots from four independent experiments (n=4) for protein expressions of Ki-67 and β-actin at different cycles (1, 3, or 5) of cell division in HSG and HSY after being repeatedly exposed to dental X-ray for 5, 10, or 20 times (x). The size of Ki-67 is as predicted; Control=non-irradiated cells. (C, D) Box plot graphs showing Ki-67 expression in HSG and HSY. The band intensities of Ki-67, quantified using Image J software, were normalized by those of β-actin. The degrees of Ki-67 expression in each experimental sample were compared to those in non-irradiated cell control (CTL) at the same cycle of cell division, set to 1. A horizontal line in each box represents the median; * $p<0.05$.

active caspase 3 band in a positive control, i.e., HSG or HSY treated with 0.1 M staurosporine for four hours (Figure 5).

Discussion

The present study explored cell reactions to low-dose dental irradiation in two different human salivary ductal cells because salivary glands are often exposed to X-radiation and sensitive or rapidly responsive to the radiation[22]. CEA and LF expressions have been previously detected in HSG[19], derived from human submandibular salivary gland, as well as in normal intercalated duct of the parotid salivary gland[18,23]. Therefore, these two markers are useful to distinguish HSG or HSY from HOKs, since their morphology may resemble that of HOKs, i.e., a polyhedral shape, whose colonies can steadily expand to form cell clusters. The degrees of CEA and LF expressions in HSG or HSY were not only significantly higher than those of HOKs, but CEA and LF were also detected only in HSG and HSY, not in HOKs, representing the characteristics of HSG and HSY as the salivary intercalated ductal cells in nature. According to the objectives of the present study to determine the proliferative and apoptotic effects of two human salivary ductal cell lines upon exposure to low-dose irradiation, comparisons of CEA or LF expression before and after exposure to low-dose irradiation in HSG or HSY were, therefore, not studied, since CEA or LF is not regarded as a biomarker for those effects.

Since the sensitivity of each cell type to X-radiation is influenced by its rate of cell division, damages from dental radiation would happen in cells with more rapid proliferation[1]. HSG with a

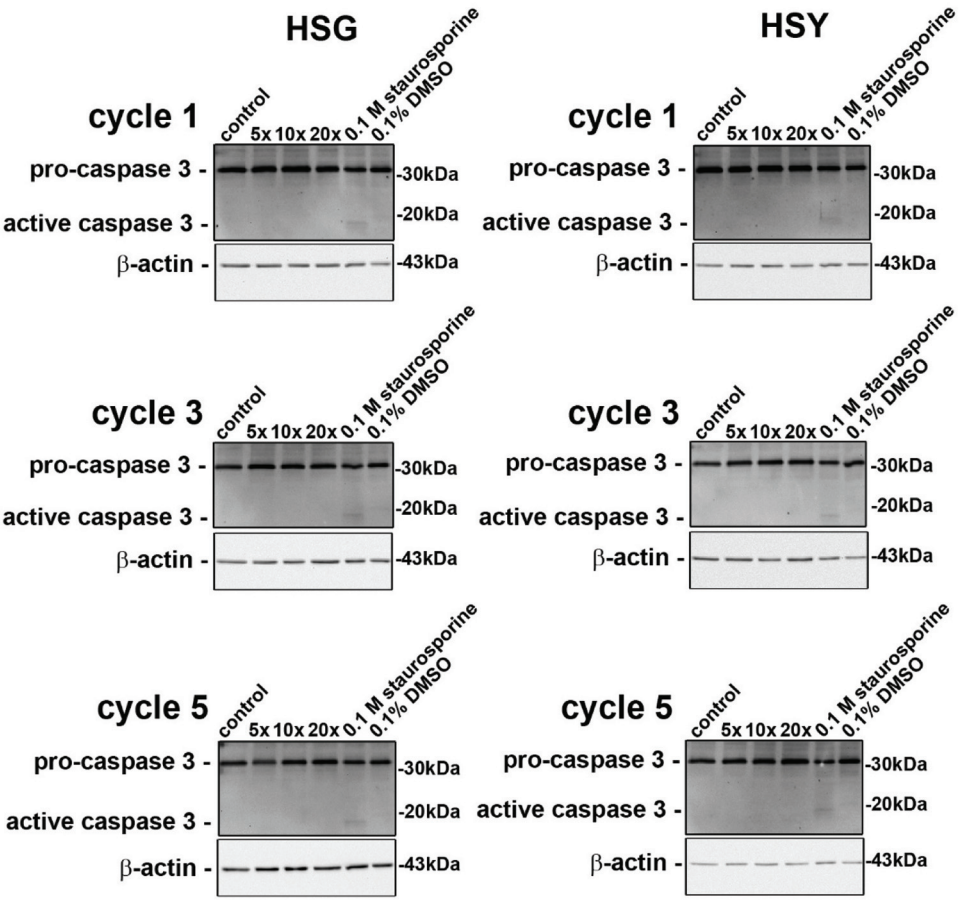


Figure 5. Representative blots from the samples in Figure 4 for constitutive expression of human pro-caspase 3 at its predicted size of 32 kDa. Note the presence of the active caspase 3 at around 17 kDa in HSG and HSY treated with 0.1 M staurosporine for 4 hours and equivalent expression of β-actin among different samples. HSG and HSY were treated with 0.1% (v/v) of dimethyl sulfoxide (DMSO) as a vehicle control for staurosporine.

faster turnover rate responded to low-dose dental X-ray by promoting transient cell proliferation at the first cycle post-irradiation relative to the control. In contrast, HSY with a slower turnover rate temporarily responded in the opposite way, indicating distinct radiosensitivity between the two cells. However, these proliferative responses to dental X-ray were reversible, as the cell proliferation rates of irradiated HSG and HSY showed no difference from those of the non-irradiated cells at or after the third cycle, suggesting that HSG and HSY have an ability to recover and resume their normal rates of proliferation.

An increase or a decrease in cell proliferation after exposure to low-dose irradiation found in HSG or in HSY, respectively, is consistent with a theory of "radiation hormesis" or adaptive responses[24]. The distinct mechanisms of radiation hormesis for increased or decreased cell proliferation in these two salivary ductal cells lines are not known, but they are likely to depend on the characteristics of various cell types. For example, high resistance to chromosome aberrations was caused by exposure to low-dose irradiation in lymphocytes, implying that a low dose of ionizing radiation promotes cellular resistance[25]. Moreover, consistent with the increased proliferation of HSG, an increased proliferative activity of a mouse osteoblastic cell line, MC3T3-E1, was observed after a single dose (500 mGy) of X-ray[26]. By contrast, the decreased proliferation, by a delay of cell cycle progression. after low-dose irradiation has been observed in human periodontal ligament cells, albeit briefly, on day 3 after dental irradiation[27]. Feinendegen and co-workers, 1987, described that low doses of ionizing radiation caused a temporary inhibition of DNA synthesis[28]. This transient inhibition would have provided a longer time for irradiated HSY to recover. Hence, a delay in cell proliferation may be considered a protective

mechanism to mitigate and to repair irradiation-induced DNA damages.

Ki-67 is an endogenous marker for dividing cells[29,30], whose expression is peaked at the M phase[21]. A previous animal study has shown a concurrent increase in the number of BrdU-labelled and Ki-67-positive neural cells upon irradiation at 100 mGy[31]. Concordantly, BrdU-labelled cells and Ki-67 protein expression were increased in HSG upon low-dose irradiation, while they were decreased in HSY. Delayed changes in the Ki-67 expression were observed, which may have been a result of the G_2 phase blockade before continuing to the M phase[32]. Due to the absence of active caspase 3 in both cells upon exposure to any doses of X-rays after observation at different cycles, these findings indicate that cell apoptosis did not occur in both salivary ductal cells upon exposure to low-dose dental X-rays.

Conclusion

In the present study, repeatedly multiple exposures to digital X-ray transiently resulted in differential proliferative responses between the two human salivary ductal cells. However, the apoptosis did not occur in these cells. Since it is difficult to apply any in vitro effects of dental X-rays on cultured salivary cells for the dysfunction of a whole intact salivary gland, it is, therefore, required to further investigate cell and tissue reactions to low-dose irradiation in vivo. Note that other biological changes in the salivary ductal cells upon exposure to low-dose X-rays also remain to be further investigated.

It is well known that high-dose irradiation from radiotherapy in the head and neck region causes salivary gland impairment, resulting in "xerostomia". The reduced amount of saliva leads to more acidic environment in the oral cavity, enhancing decalcification of the tooth structures. Thus, rampant dental caries can frequently be encountered among patients undergoing the radiotherapy. Although the doses of X-ray used in conjunction with oral diagnosis and treatment are considered low, increasing use of cone beam computed tomography in dentistry with greater exposures to radiation than the conventional dental X-ray is likely to negatively affect salivary acinar and ductal cells. Consequently, detection of transient changes in the rate of cell proliferation upon exposure to low-dose X-rays would somehow enhance an awareness of dental practitioners for the likelihood of deleterious effects of X-radiation in dentistry. Protective measures from X-radiation must be addressed, particularly for the full-mouth radiography, and the principle of "as low as reasonably achievable" exposure with greatest benefits for patients and dental treatments must be upheld.

What is already known on this topic?

Studies have previously reported deleterious effects of high-dose irradiation on major salivary glands that are often irradiated due to radiotherapy of head and neck tumors. However, the effects of low-dose X-radiation of less than 100 mGy from dental radiography on the salivary glands and cells have not yet been studied.

What this study adds?

The findings from this study demonstrate that even exposure to low-dose X-radiation can lead to detectable alterations in the salivary cell proliferation owing to adaptive responses between the two different human salivary ductal cells, depending upon their rates of cell proliferation.

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Conflicts of interest

The authors declare no conflict of interest.

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