ว ทันต จุฬาฯ 2551;31:415-25
CU Dent J. 2008;31:415-25

Original Article
บทวิทยาการ

Effects of dental radiation on the expression of apoptotic-related genes in primary human bone cells

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Abstract

Objective To investigate the effects of dental radiation on the expression of apoptotic-related genes in primary human bone cells.

Materials and methods Human bone cells were grown in 15% FBS-DMEM and irradiated with 0, 1, 2 doses of a periapical radiograph. The cytotoxicity of irradiation was investigated by MTT assay after 24 hours. The levels of apoptotic-related gene expressions, Bcl-2, Bax, Bad, Bcl-xL, caspase-3 were analyzed by reverse transcription polymerase chain reaction, four hours after irradiation.

Results No cytotoxicity was observed at the time of 24 hours after dental irradiation. The results indicated that Bax and caspase-3 significantly decreased after irradiation as compared to the control group, whereas, the expression of Bcl-2, Bad and Bcl-xL did not change significantly after dental irradiation.

Conclusion Dental radiation affects apoptotic-related gene expressions. Therefore, prescribing dental radiograph should be highly selective and the principle of ALARA (As Low As Reasonably Achievable) should be strictly followed.

(CU Dent J. 2008;31:415-25)

Key words: *apoptosis; Bcl-2 family; caspase-3; dental radiation; human bone cells*

Introduction

Periapical radiography is a commonly used radiographic technique in dental clinics because it provides several advantages among the other dental imaging modalities, such as the least distortion, the highest resolution and the lowest radiation dose patient received.[1] Exposure to ionizing radiation as low as 1 mGy could cause DNA double-strand break of fibroblast cells which determined critical molecular lesion leading to cell death.[2-6] The third quartile patient entrance dose for an intra-oral radiograph is 3.9 mGy.[7] Thus, dental radiation might cause DNA lesion of oral cells leading to cell death through apoptotic process. This hypothesis was supported by Branemark *et al.*[8] who did not recommend performing radiographic procedures immediately after implantation due to the possibility of the detrimental effect of ionizing radiation on the healing and remodeling of bone.

Radiation-induced apoptosis is ultimately executed by caspase-3 that operated through the mitochondrial-mediated pathway involving cytochrome c released from the mitochondria in order to activate caspase-3 (Figure 1).[9,10] The mitochondrial-mediated pathway of apoptosis is regulated by the Bcl-2 family. The Bcl-2 family consists of both pro-apoptotic members, such as Bax and Bad that promote mitochondria permeability and anti-apoptotic members, such as Bcl-2, Bcl-xL, Mcl-1, Bcl-W and A1 that inhibit function of pro-apoptotic members.[11] Interactions between pro-apoptotic and pro-survival members of the Bcl-2 family of proteins are decisive in the initiation of mitochondria pore opening.[12] The members of Bcl-2 family could form either homodimers or heterodimers, suggesting neutralizing competition between these proteins.[11] Bax inactivates Bcl-2 proteins through heterodimerization and the ratio of Bax to Bcl-2 proteins increases during the apoptosis induction.[13-15] Bad forms heterodimer

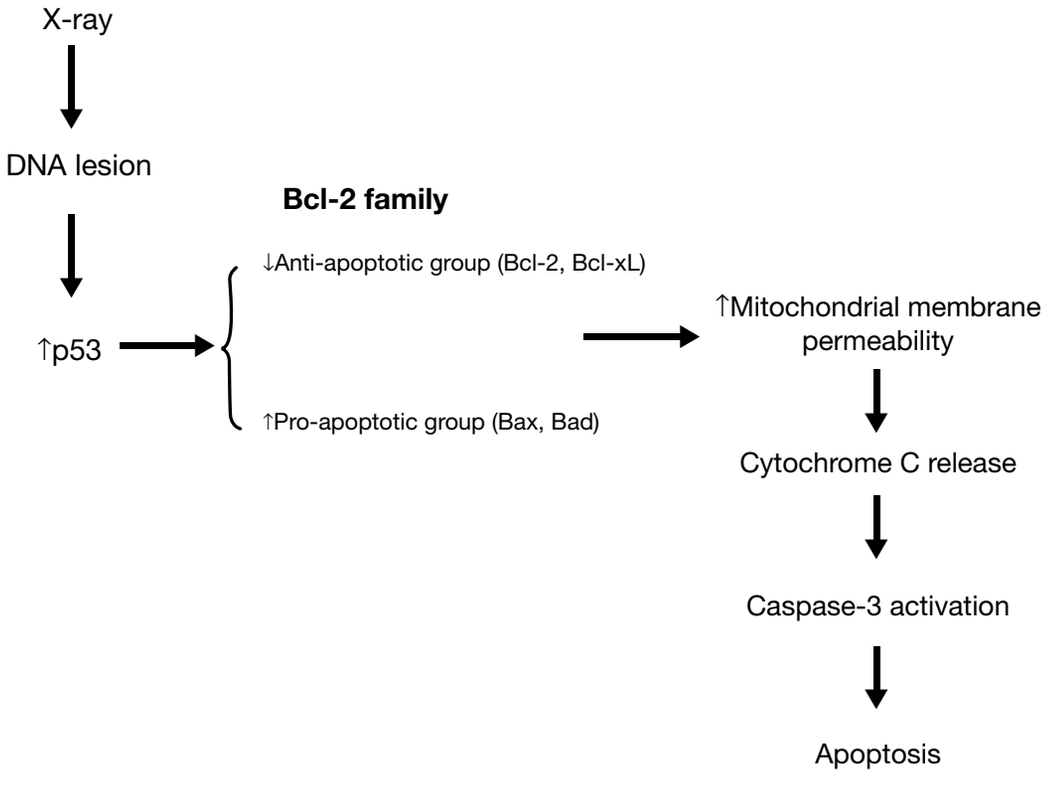

Fig. 1 Diagram of radiation-induced apoptosis pathway. Radiation-induced DNA lesions initiate apoptosis via p53-dependent mechanism, e.g. by regulating the expression of Bcl-2 family members which determine the release of cytochrome c from mitochondria resulting in caspase-3 activation.

with Bcl-xL and Bcl-2 during apoptotic process.[11,16]

Most of the studies revealed the effect of high dose radiation (> 1 Gy) on normal bone cells in a dose dependent manner including the reduction of bone cell proliferation and the synthetic activity as well as the increase of cytotoxicity and markers of cellular apoptosis and differentiation.[17-19] However, there is no scientific data revealing the biological effects of dental radiation on bone cells.

The purpose of this study was to determine the effects of short term toxicity of dental radiation on human bone cell as well as the apoptotic-related gene expression.

Materials and methods

Cell culture

Human bone cells were isolated from the fragments of alveolar process attached to the 3rd molar from patients undergoing surgery for impaction removal. The protocol was approved by the Ethical Committee, Faculty of Dentistry, Chulalongkorn University. Informed consent was received from all patients. Alveolar process fragments were dissected under sterile conditions, cut into very small pieces and washed several times in calcium and magnesium-free phosphate-buffered saline (PBS), followed by a final wash in complete Dulbecco's modified Eagles medium (DMEM), containing 15% fetal bovine serum (FBS), penicillin G (100 U/ml), streptomycin sulfate (100 µg/ml), amphotericin B (25 µg/ml) and L-glutamine (2 mM). The bone fragments were cultured in complete DMEM, at 37°C in 95% air/5% CO_2. Cells were allowed to craw out and grow on the culture plate. After the culture reached the confluent state, cells were removed from the plate and seeded in a new vessel with 1:3 ratio. All of the experiments were performed using cells from passage 3rd-5th. Cells were seeded at a density of 40,000 cells/well in a 24 well-plate or 400,000 cells/dish in 35 mm tissue culture dishes for MTT assay and RNA isolation, respectively.

Cell irradiation

The cultures were irradiated with 0, 1, 2 doses using a dental x-ray machine generator (Gendex 1000, Gendex Corp., IL, USA). The radiographic parameters for one dose were 75 kVp, 15 mA, and 0.26 sec. The focal-object distance was 8 inches. Four hours after irradiation, total RNA was isolated and reverse transcription polymerase chain reaction (RT-PCR) assay was performed to determine the expression levels of Bcl-2, Bax, Bad, Bcl-xL and caspase-3. For cytotoxicity, cells were evaluated using MTT assay 24 hours after irradiation.

MTT assay

The MTT assay is based on the reduction of the yellow tetrazolium salt to insoluble purple formazan crystals by dehydrogenase enzymes from the mitochondria of metabolically active cells. The amount of purple formazan crystals formed is proportional to the number of viable cells. First, cells were incubated with MTT solution 350 µl (0.5 mg/ml in DMEM without phenol red) for 30 minutes at 37°C. The solution was then removed and a buffer solution containing dimethylsulfoxide (DMSO) and glycine buffer (pH = 10) was added to dissolve the formazan crystals. The purple of formazan in solution was quantitated by colorimetric assay, using spectrophotometer (Genesys UV scanning, Thermospectronic, Roche, NY, USA), using the absorbance at 570 nM and converted into the cell number by comparing with the standard curve established from the culture of known cell number. All measurements were done in triplicate.

Reverse transcription-polymerase chain reaction

Total RNA was extracted from irradiated human bone cells using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) according to

manufacturer's instruction. The concentration of RNA was determined using a UV-spectrophotometer. One microgram of total RNA from each sample was used to generate cDNA by using Reverse transcription kit (Promega, Madison, WI, USA). Subsequently, polymerase chain reactions were performed using tag polymerase (Invitrogen, Brazil) by a thermocycler (Tpersonal, Whatman Biometra, Goettingen, Germany) to detect the expression level of Bcl-2, Bax, Bad, Bcl-xL, caspase-3. The expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The PCR products were analyzed by electrophoresis in 2% agarose gel and visualized by ethidium bromide fluorostaining. The band intensity was quantified using Scion Image software (Scion, Frederick, MD, USA). Table 1 showed the sequences of primer pair used in this study. The reaction products were amplified using the following primer pairs.

Statistical analysis

Results were expressed as mean ± SD. Statistical differences between control and irradiated groups were determined using paired t-test. A *p*-value of less than 0.05 was considered statistically significant.

Results

Twenty four hours after dental irradiation, no cell cytotoxicity was observed as shown in Figure 2. In addition, a slight increase in cell number was found in cells receiving dental irradiation.

Dental irradiation was also affected the expression of apoptotic-related genes. RT-PCR analysis in human bone cells indicated the significant reduction of Bax after dental irradiation with both 1 and 2 doses, while the reduction of caspase-3 was seen after 2 doses (Figure 3). The average in expression of most pro-apoptotic genes, such as Bax, Bad and caspase-3 were reduced. Slight decrease of Bcl-2 was also noticed.

The results from Table 2 showed the individual response of cells prepared from 6 different patients. The average Bax/Bcl-2 ratio after 0, 1 and 2 doses of dental irradiation were 1 ± 0.0, 0.98 ± 0.31 and 0.82 ± 0.12, respectively. However not all the cell lines responded in the same fashion. Cell line established from patient number 4 showed an increase ratio of Bax/Bcl-2 instead of a decrease. Similarly, the individual data from caspase-3 analysis demonstrated both up-regulation and down-regulation following dental irradiation. Eventhough the number of cell lines with the decreased expression was higher than that of the increased expression, these results indicated the variable responses from patient to patient.

Discussion

Our study revealed a new finding of the effect of dental radiation in the mGy range on primary human bone cell. The results indicated that dental radiation, the lowest diagnostic dose that would be expected not to have any influences on cell behavior, was not toxic to primary bone cells as determined by MTT assay after 1 and 2 doses of irradiation. However, we found that dental irradiation could affect the expression of apoptotic-related genes, especially Bax and caspase-3.

Interestingly, we noticed a slight increase in the MTT value in cells receiving dental irradiation. There were studies showing that low dose radiation (< 0.05 Gy) could induce cell proliferation in a dose dependent manner in various cell types such as human lung fibroblast, normal human diploid cells, Chinese hamster fibroblasts, neuron cells, and hematopoietic cells.[20-24] However, it is still not clear whether dental irradiation could affect cell proliferation. Time dependent study is required to clarify this issue.

Table 1 Primer sequences used in PCR

Gene	Sense (5′-3′) Antisense (5′-3′)	Expected product length (bp)	PCR cycle	NCBI-BLAST accession
Bcl-2	AGG AAG TGA ACA TTT CGG TGA C GCT CAG TTC CAG GAC CAG GC	126	35	NM_000633.2
Bax	TGC TTC AGG GTT TCA TCC AG GGC GGC AAT CAT CCT CTG	152	35	NM_138765.2
Bad	GAG TGA GCA GGA AGA CTC CAG C TCC ACA AAC TCG TCA CTC ATC C	320	35	NM_032989.1
Bcl-xL	TTA CCT GAA TGA CCA CCT A ATT TCC GAC TGA AGA GTG A	166	35	NM_138578.1
caspase-3	CAA ACT TTT TCA GAG GGG ATC G GCA TAC TGT TTC AGC ATG GCA C	240	27	NM_032991.2
GAPDH	TGA AGG TCG GAG TCA ACG GAT TCA CAC CCA TGA CGA ACA TGG	375	22	NM_002046

Table 2 The individual changes of Bax/Bcl-2 ratio and caspase-3 expression

Dental irradiation	Apoptotic indicator	Patient number						Average ± SD
		1	2	3	4	5	6	
1 dose	Bax/Bcl-2	0.91	1.13	0.49	1.43	1.07	0.88	0.98 ± 0.31
	caspase-3 expression	0.85	1.03	0.73	1.12	0.39	0.89	0.83 ± 0.23
2 doses	Bax/Bcl-2	0.86	0.89	0.68	1.04	0.77	0.79	0.83 ± 0.12*
	caspase-3 expression	0.61	0.61	0.51	0.88	0.35	1.06	0.67 ± 0.39*

*Statistically different, $p < 0.05$

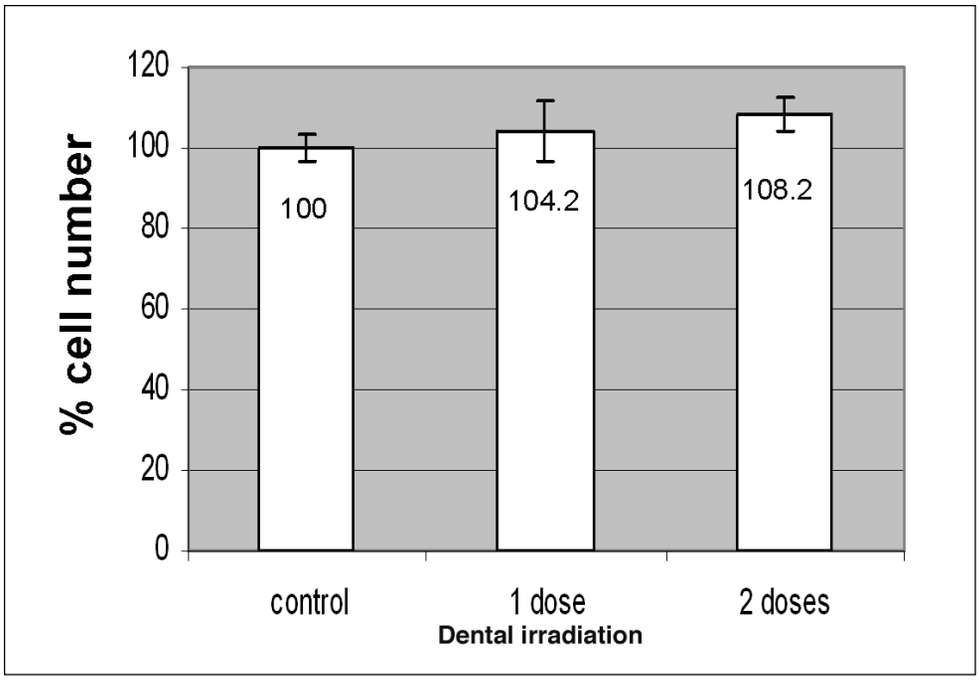

Fig. 2 The cytotoxicity of dental irradiation on primary bone cells. Cells were irradiated with 1 or 2 doses of dental radiation and the cell number was determined at 24 hour using MTT assay. Data are shown as mean ± SD (n = 3).

The significant decrease of Bax expression observed in this study might involve p53 protein, a tumor suppressor gene product whose function is involved in cell cycle arrest, apoptosis, DNA repair, and senescence following irradiation.[25-27] It is widely accepted that the Bax promoter contains a typical p53 binding site and that p53 exerts its role as inducer of apoptosis partly by trans-activating the expression of Bax gene.[28] The recent studies demonstrated that p53 accumulation was not observed after irradiation with low dose radiation (< 0.5 Gy).[20,22] Thus, the question still remains whether the mGy range dental radiation has any effect on p53 activation and, as a result, causes the down-regulation of Bax.

Radiation-induced apoptosis is a caspase-dependent mechanism. Among the effector caspases, caspase-3 plays an important role in the apoptotic process.[29,30] The reduction of caspase-3 mRNA expression indicated that dental radiation could reduce the apoptotic cell death. The previous study showed the reduction of caspase-3 activation was observed when the level of p53 was low.[31] The reduction of caspase-3 expression in our study might involve the low accumulation of p53. Future study to examine the expression of p53 is required to clarify the relationship between p53 and dental radiation.

Another important apoptotic indicator is the Bax/Bcl-2 ratio. The increased ratio of Bax/Bcl-2 in a given cell determines its susceptibility to apoptosis.[32,33] In addition, Salakou *et al.* revealed that the increased Bax/Bcl-2 ratio resulted in the up-regulation of caspase-3 and increased apoptosis.[34] It has been suggested that the Bax/Bcl-2 ratio may be more important than either promoter alone in determining apoptosis.[35] In this study, only the reduction of Bax was statistically significant, resulting in the significant decrease of Bax/Bcl-2 ratio.

The expression of Bcl-xL was also investigated in this study. It has been reported that the Bax/Bcl-2

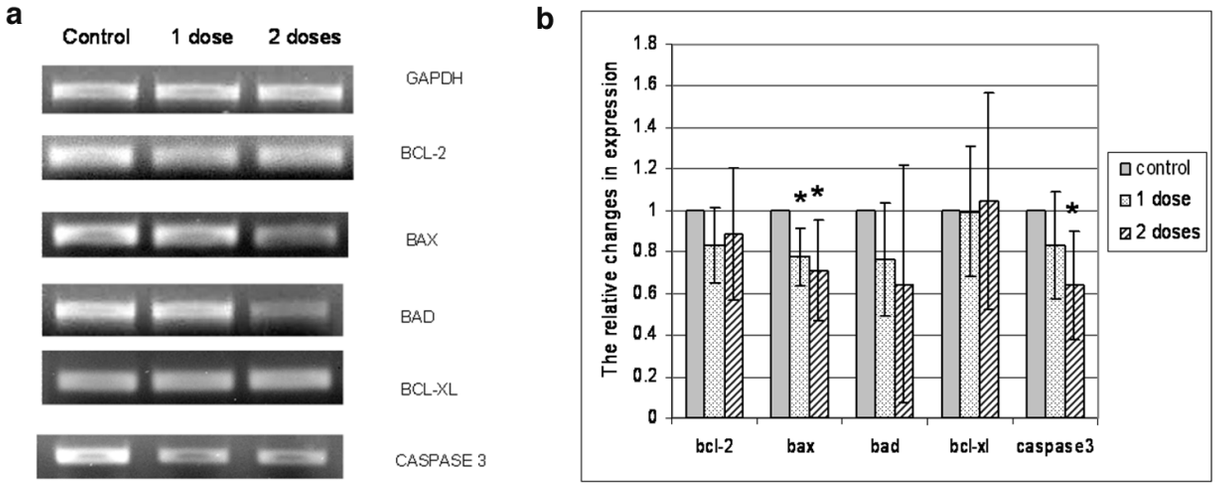

Fig. 3 The effect of dental irradiation on the expression of apoptotic-related genes. Primary bone cells were irradiated with 1 or 2 doses of dental radiation. Expression of apoptotic-related genes (Bcl-2, Bax, Bad, Bcl-xL, and caspase-3) were evaluated using a reverse transcription-polymerase chain reaction (RT-PCR) 4 hours after irradiated using the expression of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as a control.

(a) PCR images of GAPDH, Bcl-2, Bax, Bad, Bcl-xL, and caspase-3 expression from a representative experiment. (b) The quantified expression levels relative to the level of GAPDH mRNA expression. Data are illustrated as mean ± SD (n = 6). *Statistically significant, $p < 0.05$

ratio might not be used as apoptotic indicator when high expression of Bcl-xL protein was observed.[36] However, no significant change in Bcl-xL expression was noticed indicating that Bcl-xL might not be the target of dental irradiation.

Interestingly, the effect of dental diagnostic radiation could be differently observed on different primary bone culture. All bone cultures illustrated the changes on apoptotic-related genes following 4 hours irradiation. Although most of the preparations showed the down-regulation in both pro-apoptotic and anti-apoptotic genes, a few preparation of bone cell line showed different results. The difference in the response to low dose radiation may be due to either the genotypic difference of the doners or the difference in the osteoblastic stages of differentiation. Recent studies proposed that radiosensitivity may be a genotype-dependent.[37,38] It is reasonable to consider that the clonogenic survival, radiation-induced apoptosis and radiation-induced redistribution in the cell-cycle vary among the different cell line.

Although our results showed both increased and decreased expression after receiving a low dose radiation. The overall results showed the positive effect since the reduction of apoptotic-related genes in primary human bone cells outweighed the up-regulation. The results are in agreement with Feinendegen[39], who proposed that low dose radiation in the mGy range caused dual effect on cellular DNA. One is the direct DNA damage, increasing with the proportion of the dose. The other is the adaptive protection against DNA damage, depending on cell type, species and metabolism. The damage prevention may due to the increased level of antioxidant which assists to decrease reactive oxygen species (ROS) produced from radiation effects such as glutathione, superoxide dismutase after low dose irradiation.[39,40]

Therefore, dental irradiation might increase antioxidant level as a result of bio-positive effect in irradiated primary bone cells.

In conclusion, no short term toxicity of dental radiation was observed in irradiated human bone cells. Moreover, dental radiations could induce apoptotic-related gene expressions both anti-apoptotic group and pro-apoptotic group 4 hours after irradiation. The response to low dose radiation might be a genotype-dependence. Thus, prescribing dental radiographs should be highly selective based on the principle of ALARA (As Low As Reasonably Achievable).

Acknowledgements

The authors thank all the staffs in the Research Unit of Mineralized Tissue in Faculty of Dentistry, Chulalongkorn University for their help and useful suggestions. This study was supported by the grant from Graduate School, Faculty of Dentistry, Chulalongkorn University and Thailand Research Fund (master research grant).

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ผลของรังสีทันตกรรมต่อการแสดงออกของยีนที่เกี่ยวข้องกับการขจัดตัวเองของเซลล์ในเซลล์เพาะเลี้ยงจากกระดูกของมนุษย์

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บทคัดย่อ

วัตถุประสงค์ เพื่อศึกษาถึงผลของรังสีทันตกรรมต่อการแสดงออกของยีนที่เกี่ยวข้องกับการขจัดตัวเองของเซลล์ในเซลล์เพาะเลี้ยงจากกระดูกของมนุษย์

วัสดุและวิธีการ เซลล์กระดูกของมนุษย์ได้รับการเพาะเลี้ยงในอาหารเลี้ยงเซลล์ดีเอ็มอีเอ็มที่มีซีรัมร้อยละ 15 และได้รับการฉายรังสีในปริมาณ 0 1 2 เท่าของปริมาณรังสีที่ใช้ในการถ่ายภาพรังสีชนิดรอบปลายราก ภายหลังการฉายรังสี 24 ชั่วโมงตรวจสอบอันตรายของรังสีต่อเซลล์กระดูกโดยวิธีเอ็มทีที วิเคราะห์ระดับการแสดงออกของยีนที่เกี่ยวข้องกับการขจัดตัวเองของเซลล์ ได้แก่ บีซีแอลทู แบกซ์ แบด บีซีแอลเอ็กซ์แอล คาสเปสทรี โดยวิธีอาร์ทีพีซีอาร์ ภายหลังการฉายรังสี 4 ชั่วโมง

ผลการศึกษา รังสีทันตกรรมไม่มีอันตรายต่อเซลล์เพาะเลี้ยงจากกระดูกของมนุษย์ที่เวลา 24 ชั่วโมงหลังการฉายรังสี ผลการศึกษาพบว่าการแสดงออกของยีนแบกซ์และคาสเปสทรีลดลงอย่างมีนัยสำคัญภายหลังการฉายรังสีทันตกรรมเมื่อเปรียบเทียบกับกลุ่มควบคุม ในขณะที่ไม่พบการเปลี่ยนแปลงการแสดงออกของ บีซีแอลทู แบด บีซีแอลเอ็กซ์แอลอย่างมีนัยสำคัญหลังการฉายรังสีทันตกรรม

สรุป รังสีทันตกรรมมีผลต่อการแสดงออกของยีนที่เกี่ยวข้องกับการขจัดตัวเองของเซลล์ ดังนั้นการสั่งถ่ายภาพรังสีทางทันตกรรมควรคำนึงถึงอันตรายจากรังสี โดยยึดหลักให้ผู้ป่วยได้รับปริมาณรังสีน้อยที่สุดเท่าที่จะเป็นไปได้

(ว ทันต จุฬาฯ 2551;31:415-25)

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