RESEARCH ARTICLE

Low-dose radiations derived from cone-beam CT induce transient DNA damage and persistent inflammatory reactions in stem cells from deciduous teeth

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Objectives: Cone-beam CT (CBCT), a radiographic tool for diagnosis, treatment, and follow-up in dental practice, was introduced also in pediatric radiology, especially orthodontics. Such patients subjected to repetitive X-rays examinations may receive substantial levels of radiation doses. Ionizing radiation (IR), a recognized carcinogenic factor causing DNA double-strand breaks (DSBs) could be harmful to undifferentiated cells such as dental pulp stem cells (DPSCs) since inaccurately repaired or unrepaired DSBs may lead to malignant transformation. The H2AX and MRE11 proteins generated following DSBs formation and pro-inflammatory cytokines (CKs) secreted after irradiation are relevant candidates to monitor the cellular responses induced by CBCT.

Methods: DPSCs were extracted from human exfoliated deciduous teeth and their phenotype was assessed by immunocytochemistry and flow-cytometry. Cells were exposed to IR doses: 5.4–107.7 mGy, corresponding to 0.5–8 consecutive skull exposures, respectively. H2AX and MRE11 were detected in whole cells, while IL-1 α , IL-6, IL-8, TNF α in supernatants, using enzyme-linked immunosorbent assay (ELISA) at different time points after exposure.

Results: The phosphorylation level of H2AX in DPSCs increased considerably at 0.5 h after exposure ($p < 0.001$ for 3, 5, 8 skull exposures and $p < 0.05$ for 1 skull exposure, respectively). MRE11 response could only be detected for the highest IR dose ($p < 0.001$) in the same interval. CKs secretion increased upon CBCT exposure according to doses and time.

Conclusions: The DPSCs exposure to CBCT induces transient DNA damage and persistent inflammatory reaction in DPSCs drawing the attention on the potential risks of IR exposures and on the importance of dose monitoring in pediatric population.

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Introduction

Cone-beam CT (CBCT) is a multipurpose radiographic tool for diagnosis, treatment planning, and follow-up in dental practice. This procedure provides three-dimensional information at a relatively high resolution with considerably lower radiation dose [mean adult effective doses are 212 μ Sv for large field-of-view (FOV), 177 μ Sv for medium FOV, and 84 μ Sv for small FOV] than conventional multislice CT (0.5–1.4 mSv for unenhanced head CT) but remain higher than those derived from conventional dental X-ray imaging (8.1 μ Sv).^{1,2} CBCT was introduced also in dental pediatric radiology, such as in the field of orthodontics. A typical orthodontic patient is subjected to several conventional or two-dimensional radiographs: cephalometric, panoramic, and intra-oral, therefore due to repetitive CBCT scans radiation doses might reach important levels.^{3,4}

Ionizing radiation (IR) is a known carcinogen;⁵ however, the carcinogenic risk at low doses (below 100 mSv) is controversial and needs to be thoroughly investigated. Concerns about radiation exposure are greater for children because they are more sensitive to malignancies induced by exposure to ionizing radiation as compared to adults.^{6–8} In the recent years, several studies evaluated the risk related to low-dose radiation exposure, most of them being epidemiological studies.^{9–11}

Human mesenchymal stem cells (hMSCs) were defined by the International Society of Cellular Therapy based on their adherence to plastic surface, expression of markers, such as CD73, CD90, and CD105, and lack the expression of others, such as CD45, CD34, CD14, CD79 α , CD19, and HLA-DR. The hMSCs are capable of differentiating into osteoblasts, chondroblasts, and adipocytes under appropriate *in vitro* conditions.¹² The clarification of whether these cells are implicated in radiogenic cancers induction would be a major point for scientists to address. An alternative source for hMSCs are the dental pulp stem cells (DPSCs) which have shown to play a crucial role in tooth development and regeneration processes following dental trauma.^{13,14}

IR produces DNA damage directly and indirectly by the ionization of water and production of free radicals which can damage DNA, proteins, and lipids.¹⁵ DNA damages caused by IR are single-strand breaks or double-strand breaks (DSBs), the latter if not repaired, can lead to cell death. Alternatively, inaccurately repaired or unrepaired DSBs may result in mutations or genomic rearrangements in a surviving cell, which in

turn can lead to genomic instability and malignant cell transformation.¹⁶

H2AX is a variant of H2A histone proteins family, representing ~10% of it, randomly incorporated throughout the DNA. The formation of DSBs is always followed by the phosphorylation of H2AX, usually on Ser 139 residue, the resulted molecule being termed as γ H2AX. The PI3K pathway molecules, such as ATM (ataxia telangiectasia mutated), ATR (ATM-Rad3-related), and DNA-PK (DNA-dependent protein kinase) are responsible for this process.¹⁷

After phosphorylation repair proteins are attracted such as the MRN complex, consisting of MRE11 (human meiotic recombination 11 protein), RAD50, and NBS1 (defective gene responsible for the Nijmegen breakage syndrome), this complex is implicated in the detection of DSBs and the recruitment of ATM to the site of the damage.¹⁸ ATM targets substrates such as BRCA1 (breast cancer protein 1), 53BP1 and 53BP2 (p53 binding proteins 1 and 2), and MDC1 (the mediator of DNA damage checkpoint), as well as the checkpoint proteins, Chk1 and Chk2.¹⁹

These processes are directed to stop the progression of the cell cycle and to activate proteins responsible for DNA repair, thus promoting genome stability.²⁰

Cytokines (CKs), soluble “factors” secreted by various cell types after stimulation, have major relevance also in radiation research.^{21,22} Specifically, pro-inflammatory CKs as components of the early response programs to irradiation, contribute to the immune system’s modulation and increased expression of proto-oncogenes and other CKs.²³ Anti-oxidant and anti-inflammatory CKs then act to restore homeostasis, the pro-inflammatory phase persisting until the apparent challenges to the host integrity are eliminated. CKs production is time-dependent, peaking usually at 4–24 h after irradiation with subsequent decrease to baseline levels within few days.²⁴ Inflammatory reactions induced by irradiation are mediated most frequently by IL-1 α , IL-6, IL-8, TNF α .^{25,26}

The present study arose from the necessity to evaluate the radiation risk in pediatric patients exposed to repeated CBCT examinations. The radiosensitivity of hMSCs was examined in few studies, primarily focused on bone-marrow derived cells exposed to high doses of radiation^{27,28} warranting further research. The evaluation of the effects of low-dose radiation on human DPSCs biology in order to provide basic information

on possible side effects of CBCT examinations on tooth development and/or regeneration is of paramount importance. The main aim of such studies is the identification and characterization of the role of DNA damage and its repair process following clinically relevant irradiation schedules. On the other hand inflammatory CKs produced following IR exposure which mediate and regulate several cellular processes, such as protein synthesis, cell growth, and apoptosis, may impact upon DNA damage/repair processes, as well. Therefore, it is essential to ascertain the effects and potential risks of clinically relevant IR doses in children, specifically in stem cells originated from teeth, which are directly irradiated during CBCT examination. The novelty of the present study relies both in the assessment of some particular molecules (γ H2AX, MRE11, and inflammatory CKs IL-1 α , IL-6, IL-8, TNF α) induced by CBCT exposure in human DPSCs, but also in the selected methodology *i.e.* cell-based enzyme-linked immunosorbent assay (ELISA) *vs* fluorescence microscopy, in which the cellular responses are quantified per sample instead of per cell basis.

Methods and materials/patients

Establishment of the DPSCs cultures from an exfoliated deciduous teeth

The exfoliated deciduous teeth were obtained from an 11-year-old boy, with informed and oral consent from the patient and parents according to the ethical guidelines set by the French law and with a dedicated authorization for this work (no. DC-2009-927, Cellule Bioéthique DGRI/A5, Paris, France). The DPSCs were isolated as previously reported.^{13,27,28} Briefly, after decontamination with povidone-iodine solution, teeth were sectioned and the exposed pulp tissues were collected and enzymatically digested with Type I collagenase and dispase. Cells were then seeded at a density of 104 cm⁻² and the cultures were maintained with Dulbecco's Modified Eagle Medium 1 g l⁻¹ D-Glucose (Sigma-Aldrich, MO), supplemented with 10% Fetal Bovine Serum (Sigma-Aldrich, MO), 1% Penicillin/Streptomycin (Sigma-Aldrich, MO), at 37°C with 5% CO₂. Cells were detached by trypsinization at 70–80% confluence and either replated at the above mentioned density or frozen at –80°C. For all experiments, cells were used between passages 2 and 4. Their phenotype was confirmed by immunocytochemistry staining and flow-cytometry analysis.

Cultivation and irradiation of the DPSCs

Cells harvested at a cell population density of 15 × 10³/well were incubated 24 h in the cell culture conditions described above. Some plates were kept as control (non-irradiated) and others were subjected to irradiation within the dose range of 5.4–107.73 mGy. After irradiation, the cell culture media was collected at 0, 6,

Figure 1 Polymethyl methacrylate phantom used for the irradiation of the cells (a). The 96-well-plate containing the stem cells at the height corresponding to the location of the oral cavity (b). The phantom with the 96-well-plate placed in Planmeca 3D ProMax CBCT device used for irradiation (c). CBCT, cone-beam CT.

and 24 h and kept at –70°C until the assessments of the inflammatory CKs. For the determination of H2AX and MRE11 proteins, the irradiated cells were fixed with fixation solution available in the ELISA kits immediately after CBCT irradiation (at 0 h), at 0.5 h, and at 24 h after that and maintained in fixation solution until processing at 4°C.

These time points were chosen according to the literature data which show the highest inflammatory CKs production between 4–24 h,^{23,24} and the most significant H2AX phosphorylation at ~0.5 h after exposure to IR,^{29,30} while MRE11 induction is expected right after γ H2AX foci formation.^{19,20} Additionally, a preliminary study conducted on DPSCs exposed to the same CBCT doses showed that later than 0.5 h after the CBCT exposure significant changes occurred only for the highest dose (107.73 mGy) (Supplementary material) which are not used in the clinical practice. The aim of the study was to evaluate the changes induced by clinically relevant doses (*i.e.* 12.83 mGy derived from 1 full skull exposure), consequently the time frame was chosen accordingly.

A cylindrical polymethyl methacrylate phantom (23 × 16 cm), corresponding to a 10-year-old child's head was used for the irradiation of the cells (Figure 1a). A specific hole was created for the 96-well-plate containing the stem cells at the height of 15 cm from the bottom, corresponding to the location of the oral cavity (Figure 1b). The Planmeca 3D ProMax (Planmeca, Finland) CBCT device was used for irradiation (Figure 1c). The skull scanning protocol was: FOV 230 × 260 mm, 96kV, 9mA, 27 s, DAP 3462 mGy*cm², voxel 600 μ m. Radiation doses were measured with thermoluminescent dosimeters placed inside of the phantom, in the same position where the stem cells were placed. The different doses of CBCT were obtained by repeated exposure of the phantom using the same settings. Consequently, the following doses were measured and tested: 5.4 mGy, corresponding to 0.5 skull exposure (mandibular jaw exposure); 12.83 mGy, corresponding to 1 full skull exposure; 33.95 mGy, corresponding to 3 consecutive skull exposures; 65.99 mGy, corresponding to 5 consecutive skull exposures; and 107.73 mGy, corresponding to 8 consecutive skull exposures. For the

determination of H2AX and MRE11 proteins all the above mentioned doses were tested. The inflammatory CKs production was evaluated after 1 skull exposure, the most frequently used procedure in the clinical practice, which corresponds to 12.8 mGy. Aiming to obtain significant results a higher CBCT dose (107.73 mGy) was also tested.

Immunocytochemistry staining

The cells were seeded at a density of 3×10^4 cells in 200 μ l medium/well in 96-well-plates. Upon reaching subconfluency, the cells were washed three times with Phosphate Buffered Saline (PBS) and fixed with 4% paraformaldehyde solution in PBS for 20 min at room temperature. A further step of permeabilization was performed with 0.1% Triton X-100 in PBS for 20 min, followed by blocking of unspecific antibody bound with 10% Bovine Serum Albumin in PBS, 20 min at room temperature. Staining was performed for the following stem cell markers: CD44 FITC (fluorescein isothiocyanate) (Becton Dickinson), dilution 1:20; CD73 PE (phycoerythrin), SSEA-4 (stage-specific embryonic antigen-4), ALP (alkaline phosphatase), Sox-2 (SRX (sex determining region Y)-box 2), and Oct3/4 (octamer-binding transcription factor 4) (Santa Cruz Biotechnologies, Santa Cruz, CA), dilution 1:50; and Homebox protein Nanog (R&D Systems), dilution 1:50. Samples were incubated with the primary antibodies overnight at 4 °C in dark, followed by incubation 45 min at room temperature with secondary antibody goat anti-mouse IgG labeled with FITC (Santa Cruz Biotechnologies) (1:50). After each step the cells were washed with PBS. For nuclei staining the slides were mounted with 4,6-diamidino-2-phenylindole (DAPI) (Santa Cruz Biotechnologies) and image acquisition was performed with a Zeiss Axiovert microscope using filters at 488, 546, and 340/360 and an AxioCam MRC camera.

Flow-cytometry analysis

For each sample, 10×10^5 cells were stained with CD73 PE, CD105 PE, CD29 PE, CD49e PE, CD166 PE, CD117 PE (Santa Cruz Biotechnologies), dilution 1:50; CD 34 FITC, CD44 FITC, CD45 FITC (Becton Dickinson), dilution 1:20; as well as with negative controls IgG1, FITC, and IgG1 PE (Sigma-Aldrich). The incubation with the monoclonal antibodies for 60 min at 4 °C in dark was followed by washings with cold PBS. Stained cells were analyzed with a FACS Canto II, 6-color flow-cytometer (Becton Dickinson) using FACS Diva v. 6.1.3 software (Becton Dickinson).

Determination of histone H2AX, MRE11, and inflammatory CKs with ELISA

A cell-based ELISA assay with fluorogenic substrate was used to measure total (H2AX) and phosphorylated (γ H2AX) proteins in whole cells (R&D Systems, Abingdon, UK). The method was performed according to the manufacturer's indications, measuring the target

protein's phosphorylation status using a double immunoenzymatic labeling procedure. A phospho-specific antibody and a normalization antibody were used in order to assess the proteins according to their phosphorylation status. Measurements were performed with the BIOTEK Synergy 2 microplate reader (Winooski, VT) operated in fluorescence mode, at 600 nm for the phosphorylated H2AX and at 450 nm for the total H2AX. γ H2AX/total H2AX fluorescence ratios were calculated in accordance to the manufacturer's recommendations.

A colorimetric cell-based ELISA was used to assess the MRE11 protein's expression profile in whole cells by the measurement of its relative amounts (Assay Biotechnology Company, CytoGlow, Sunnyvale, CA). Determinations were done according to the manufacturer's indications. After incubation with specific primary and secondary antibodies the target protein was measured with BIOTEK Synergy 2 microplate reader (Winooski) operated in absorbance mode, at 450 nm wave length.

Inflammatory CKs (IL-1 α , IL-6, IL-8, and TNF α) were evaluated from the cells' supernatants using sandwich ELISA technology (Abbexa Ltd, Cambridge, UK). Determinations were done according to the manufacturer's indications and measurements were performed with the TECAN Sunrise microplate reader (Grödig/Salzburg, Austria) in absorbance, at 450 nm wavelength.

Statistical analysis

Statistical data processing was done using GraphPad-Prism software program, v. 5.0 (GraphPad, San Diego, CA). Statistical comparisons between groups were made by the Unpaired *t*-test ($p < 0.05$ statistical significance).

Results

Immunocytochemistry and flow-cytometry analysis confirm the stemness of the DPSCs

Immunocytochemistry demonstrated a pluripotent stem cell phenotype, with both mesenchymal and embryonic characteristics. The cells showed strong positivity for transcription factors implicated in the maintenance of embryonic stem cells pluripotency (Sox-2, Nanog, and SSEA-4), as well as for mesenchymal stem cells markers (CD44, CD73, Oct3/4, and ALP) (Figure 2).

Cells markers

CD44, CD166, CD73, CD49e, CD29, and CD105, whereas the cells were negative (0.1–0.2%) for CD34 (early hematopoietic and vascular-associated tissue), CD45 (common leukocyte), and CD117 (hematopoietic stem cells) markers (Figure 3).

Immunocytochemistry and flow-cytometry analysis performed at the same passages²⁻⁴ when the CBCT irradiation experiments were done confirmed the stemness of the DPSCs.

Figure 2 Immunocytochemical staining for embryonic stem cells pluripotency (Sox-2, Nanog, and SSEA-4) and mesenchymal stem cells (CD44, CD73, Oct3/4, and ALP) markers. .

CBCT exposures induced a dose-dependent γ H2AX response in DPSCs

The calculated γ H2AX/total H2AX fluorescence ratios showed that in the used dose range (0.5–107.73 mGy) the exposure of the cells to CBCT-induced modifications in the phosphorylation level of the assessed protein (Figure 4). These changes appeared immediately after

CBCT exposure and culminated after 0.5 h. Differences were dose-dependent (however not linear), the most significant modifications induced immediately after CBCT exposure were registered for doses corresponding to 5 and 8 skull exposures ($p < 0.001$). At 0.5 h significant modifications were also registered for doses corresponding to 3 skull exposures ($p < 0.001$). The mGy, corresponding to mandibular jaw exposure and 1 full skull exposure, respectively), also caused differences in the H2AX phosphorylation level. However, the differences became insignificant 24 h after exposure for all the tested doses.

CBCT exposures induce a weak expression of MRE11 in DPSCs

MRE11 DNA repair protein was induced by CBCT irradiation only after the exposure to the highest dose (107.73 mGy) of the cells at 0.5 h after irradiation ($p < 0.001$) (Figure 5). For the other doses and time points of evaluation there was no relevant change in the relative amount of this protein.

CBCT exposures induce inflammatory CKs production in DPSCs

IL-1 α , IL-8, and TNF α expressions were strongly influenced by the CBCT exposure, generally in a dose- and time-dependent manner (Figure 6).

Figure 3 Flow-cytometry histograms of cells stained with monoclonal antibodies characteristic for mesenchymal stem cells.

Figure 4 γ H2AX/total H2AX fluorescence ratios in DPSCs after CBCT irradiation (at 0, 0.5, and 24 h). Values of H2AX proteins are expressed as relative fluorescence units. Statistical comparisons between groups were made by Unpaired *t* test, **p* < 0.05; ***p* < 0.001; (mean $\pm$ SEM, *n* = 3). CBCT, cone-beam CT; DPSCs, dental pulp stem cells; SEM, standard error of mean.

IL-1 α increased significantly at 6 h after CBCT exposure for both tested doses (*p* < 0.001 for 12.83 mGy and *p* < 0.0001 for 107.73 mGy, respectively); after 24 h this molecule remained elevated at a significant level when compared to control (*p* < 0.0001 for both doses).

The IL-8 level was significantly up-regulated at 6 h only by the higher CBCT-derived dose (107.73 mGy, *p* < 0.001), but after 24 h even the clinically relevant dose produced significant difference (*p* < 0.05).

The level of TNF α showed significant increase after both CBCT-derived doses at both time points tested in the present study (*p* < 0.0001 for all parameters).

IL-6 level was not affected significantly by the CBCT irradiation doses at the selected time points. The only notable difference for this CK was recorded between the non-irradiated controls, measured at 24 vs 6 h after irradiation (*p* < 0.05), which occurred most likely due to the proliferation of the cells.

Overall, the values of three of the four assessed CKs (IL-1 α , IL-8, and TNF α) remained significantly up-regulated after 24 h from CBCT irradiation as compared to controls.

Nevertheless, if the absolute values of these CKs induced by the same dose are analyzed at 6 vs 24 h after

irradiation, the tendency is decreasing in time. Thus, the absolute value of IL-1 α induced by dose 12.83 mGy decreases from 0.32 to 0.28 pg ml⁻¹ (*p* = 0.0108) and by dose 107.73 from 0.37 to 0.32 (*p* = 0.0036); the absolute value of TNF α measured for dose 12.83 mGy decreases from 0.80 to 0.53 pg ml⁻¹ (*p* = 0.0004) and for dose 107.73 decreases from 1.342 to 1.073 (*p* = 0.0002); the absolute value of IL-8 measured for dose 12.83 mGy decreases from 242.1 to 234.2 pg ml⁻¹ (not significant) and for dose 107.73 decreases from 331.1 to 296.3 (not significant) (Figure 6).

Discussion

CBCT examination is considered to be “low-dose” imaging (High Level Expert Group³¹); however, it is of major importance to identify and to quantify the effects and potential risks of clinically relevant IR doses in children, specifically in stem cells originated from teeth, and directly irradiated during CBCT examination.

Earlier studies evaluated the effects of low doses of IR in atomic bomb survivors¹¹ and occupationally exposed population⁹ while similar studies on stem

Figure 5 Expression of MRE11 protein after CBCT irradiation in DPSCs (at 0, 0.5, and 24 h). Statistical comparisons between groups were made by Unpaired *t* test, ***p* < 0.001; (mean $\pm$ SEM, *n* = 3). CBCT, cone-beam CT; DPSCs, dental pulp stem cells; SEM, standard error of mean.

Figure 6 The CKs' (IL-1 α , IL-6, IL-8, and TNF α) production after CBCT irradiation by the DPSCs (at 6 and 24 h). Statistical comparisons between groups were made by Unpaired *t* test, **p* < 0.05; ***p* < 0.001; ****p* < 0.0001 (mean $\pm$ SEM, *n* = 3). CBCT, cone-beam CT; CKs, cytokines; DPSCs, dental pulp stem cells; SEM, standard error of mean.

cells endorsed the effects of high IR doses on bone-marrow derived stem cells.^{25,26} One study assessed the effects of orthodontic radiography-derived X-rays in children measuring the number of micronucleated cells in the exfoliated oral mucosa cells. This study found this procedure cytotoxic to buccal mucosa exfoliated cells.³² Another interrelated study evaluated the DNA damage induced by X-rays with micronucleus assay and observed increased micronuclei formation in bone marrow mesenchymal stem cells.³³

The present study assessed the DNA damage/repair and the induction of inflammatory CKs in DPSCs following the exposure to low-dose IR derived from CBCT.

The phosphorylation level of the histone protein H2AX following CBCT irradiation of DPSCs was related to time and dose, however no linear dose-dependency was observed most likely due to the low and tight dose range that was tested. The peak level of γ H2AX was registered for the highest irradiation dose (107.73 mGy) at 0.5 h after CBCT exposure. This dose (resulted upon 8 consecutive CBCT exposures) corresponds to the

admitted upper limit of low doses, and it is not used in the clinical practice for diagnostic proposes. However, the IR dose corresponding to a full skull exposure (12.83 mGy), resulting from the most frequently used CBCT procedure caused significant H2AX phosphorylation, as well. It should be noted that this overexpression was transient, ceasing after 24 h from exposure.

Similar results were obtained by Tu *et al*²⁹ in HeLa cells exposed to 1 Gy IR dose: the peak number of γ H2AX foci was observed at 0.5 h after irradiation and gradually decreased afterward. For a higher dose (4 Gy), the number of γ H2AX foci reached the maximum level after 1 h, with a second peak at 8 h from exposure, but a complete DSBs repair occurred afterwards. The formation of γ H2AX foci reaching a maximum level after 10–30 min was observed by other authors, as well.^{32,33} Several studies found increased levels of γ H2AX foci at doses 1–10 mGy and a decrease in their level up to 24 h in human fibroblast cultures^{15,34} in mononuclear cells from patients exposed to X-rays derived from CT,³⁵ in pediatric patients undergoing cardiac catheterization,⁸ and in lymphocytes after coronary CT angiography.³⁶ Johnston *et al*³⁷ described a

linear correlation between the received radiation dose (dose range 0–5 Gy) and phosphorylation level of H2AX of *ex vivo* irradiated mouse embryo fibroblast cultures and human whole-blood cells. In their study, the measurements were done with a simplified ELISA, the response being quantified per sample instead of per cell basis, similar to the present study. However, they found a linear proportionality between the received radiation dose and the phosphorylation level of H2AX, this dissimilarity to our results being probably attributable to the different cell types (whole blood/mouse embryo cells vs DPSCs) and to the higher dose range used in the cited study (0–5 Gy vs 5.4–107.73 mGy).

The induction of MRE11 only by the highest dose (107.7 mGy) tested in the present study is in accordance to the results of Rothkamm and Lobrich³⁴ which demonstrated a compromised DSB repair at very low dose range (<20 mGy) in primary human fibroblasts. A recent paper demonstrated a lack of repair after the exposure of human neural progenitor cells to³¹ mGy and then the induction of homologous recombination and non-homologous end-joining repair pathways after the irradiation of the same cells with a higher dose (124 mGy).³⁸ This study sustains the appearance of linear dose-dependency between the received radiation dose and the phosphorylation level of H2AX only at doses above 124 mGy. Therefore, it is conceivable that a low range of IR doses does not induce a linear proportionality between dose and H2AX phosphorylation. Also, it may not initiate MRE11 involvement in the DNA repair mechanisms hence supporting the idea of a different behavior of MRE11 as compared to H2AX in the present study. Colin et al³⁹ observed a non-linear dose-dependent increase of DSBs and MRE11 foci formation in three cell lines with different degrees of radiosensitivity after irradiation with higher (up to 2 Gy) IR doses, which is also consistent with our results.

CKs production following IR exposure may have impact on DNA damage/repair processes, thus they are thought to be key factors in IR associated reactions. In the present study, the evaluations of the pro-inflammatory CKs were done at 6 h and 24 h when the peak secretions of CKs are expected according to literature data.^{23,24} One full skull exposure (corresponding to 12.83 mGy), the most frequently used procedure according to the CBCT clinical protocols was selected for testing. The skull exposure was repeated in order to reproduce a higher dose (107.73 mGy) and to obtain significant results. IR-induced modifications were registered for IL-1 α and TNF α , both CKs showing significant increases both at 6 and 24 h, even after the exposure of the cells to the smaller dose (12.83 mGy). Significant increase was obtained for IL-8, as well, while for IL-6 the tested IR doses did not cause any changes at the studied time points. However, when the absolute values of these CKs were compared at 24 vs 6 h for the same IR dose, decreasing tendency was observed.

Elevated levels of IL-1 α and TNF α have been found after low-dose irradiation of various human or mammalian cells by others, as well.⁴⁰ Katsura et al³⁸ demonstrated that an IR dose range of 31–496 mGy applied on human neural progenitor cells induced alterations in the interferon and cell junction pathways, suggesting inflammation. It was demonstrated that the secretion of CKs is cell-type specific and the inflammatory CKs (IL-1 α , IL-6, IL-8, and TNF α) are rather represented in the conditioned media of the irradiated cells than immunomodulatory CKs (IFN γ , IL-2, IL-3, and IL-10).⁴¹ Similar to the current findings a dose-dependent manner of CKs increase after irradiation of keratinocytes and melanocytes was described earlier by authors from the present group.⁴² In the blood cells of Hiroshima¹¹ survivors, chronically exposed to IR, transcriptional modulations of several CKs were measured, including IL-1 β , IL-2, IL-8, IL-10, IL-12 β , TNF α / β , and macrophage colony-stimulating factor. In another study,⁴³ IL-6 and IL-8 were identified in the growth medium of human dermal fibroblasts after exposure to 2 Gy, IL-6 being reported to promote γ H2AX foci formation. The increased IL-6 production, contrary to our results is most likely due to the higher IR dose (2 Gy) and to the cell types used in the cited study (human dermal fibroblasts). Another potential explanation for the different results could be that IL-6, by activating the *Jak-stat* signaling pathways, may act as pro- or anti-inflammatory cytokine, depending on the balance between the pro- and anti-inflammatory factors at a certain moment.⁴⁴ The CKs tested in the present study were shown to alter the radiosensitivity of the cells by linking DNA damage with cell fate decisions, including DNA repair, genomic instability, cell proliferation, differentiation, and death.⁴⁵ Whilst DNA damage repair mechanisms represent an important aspect in responses to high doses of IR, subtle cellular responses such as changes in the level of some inflammatory CKs may also be relevant following exposures to low doses of radiation.²¹ The persistence of the inflammatory reaction in the CBCT-exposed human DPSCs in the present study draws the attention on the potential harmful effects of CBCT. Although a single CBCT exposure might not be harmful for the exposed cells, future exposures may result in additional damages both at DNA and inflammatory CKs levels. Particularly, children have a long life expectancy thus a long time to express the oncogenic effect of radiation exposure. While radiation-induced leukemia generally occurs earlier, solid tumors occur 10–15 years after exposure. Moreover, the consequences of low-dose radiation exposure are more severe in stem cells than in differentiated cells, due to their role as a long-term reservoir for tissue regeneration. For dental CBCT, the delivered radiation dose depending on the type of apparatus that is used and the scan parameters selected, an accurate selection of these parameters will help to minimize patient radiation doses. Moreover, reducing to minimum the amount of the imagistic examinations and monitoring the level of the received IR doses, may help to avoid the

accumulation of significant doses, especially in pediatric population.

Conclusion

The range of IR doses resulted from CBCT exposures did not lead to severe and persistent alteration in the level of DNA damage markers. However, significant increases in the amounts of some pro-inflammatory CKs were induced by irradiation. These findings draw the attention on the potential risks of IR exposures, even in the low dose range, particularly due to multiple imagistic examinations during the life span. They could lead to a significant accumulation of doses, particularly hazardous in pediatric population.

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