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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CBCT-derived low dose radiation induced effects in stem cells from deciduous teeth

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Abstract

Backgound: Cone Beam Computed Tomography (CBCT) is a radiographic tool for diagnosis, treatment planning and follow-up in dental practice. P~~An orthodontic~~~~For instance, p~~atients under orthodontic treatments ~~is~~ are subjected to several and repetitive ~~radiographs~~x-rays exams, therefore radiation doses might reach ~~significant~~ substantial levels. I~~f i~~onizing radiation (IR) ~~is a known~~recognized as a carcinogenic factor~~,~~, its effects on human tissues depend~~ing~~ on the absorbed dose, the age of the patient and the nature of the ~~and the~~ exposed tissue. The latter is mainly linked to the stage of cells differentiation, which overall will determine ~~the~~ tissue radiosensitivity ~~of the tissues~~. ~~Consequently,~~ it is of paramount importance to assess the response to IR of undifferentiated cells such as human mesenchymal stem cells (hMSCs) involved in growth as well as healing process ~~is of paramount importance~~. For instance, dental ~~Concerns about radiation exposure are greater for younger patients being more sensitive to radioinduced malignancies as compared to adults. Of paramount importance for IR risk prevention strategies is the clarification of whether human mesenchymal stem cells (hMSCs) residing in organs/tissues are prone to such outcomes. Alternatives for hMSCs are the dental~~ pulp stem cells (DPSCs) are ~~cap~~able ~~of~~ to

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differentiateing in toward odontoblasts, osteoblasts, adipocytes even neuron-like cells with crucial roles in tooth development and regeneration processes. The principal main lesions induced by IR are DNA double stranded breaks (DSBs) that, if not repaired, can lead to cell death. Alternatively, inaccurately repaired or unrepaired DSBs may lead conduct to genomic instability and subsequently malignant transformation. As a consequence of DSBs, H2AX and MRE11 are generated and thus constitute relevant candidates to monitor the cellular dose response. Moreover, The formation of DSBs is always followed by the phosphorylation of H2AX, a variant of H2A histone proteins. Repair proteins such as MRE11 are implicated in the detection of DSBs and activation of DNA repair mechanisms, thus promoting genome stability. pPro-inflammatory cytokines (CKs), which are as components of early response programs are rapidly activated after tissue irradiation and contribute contribute to the immune system's modulation regulation and increased modulate the proto-oncogenes expression of proto-oncogenesare also interesting markers to follow the exposure impact on tissues and other CKs. The present study aim to evaluated the biological changes occurred in human DPSCs exposed to low-dose irradiation derived from CBCT exposure with a focus on DNA damage proteins and CKs. **Materials and methods:** DPSCs were established extracted from human exfoliated deciduous teeth and their origin phenotype was ascertained with immunocyhistochemistry and flow cytometry. Cells were exposed to a range of IR doses (5,4-107,7 mGy, corresponding to 0.5-8 consecutive skull exposures). H2AX and MRE11 were detected from in cellular lysates, while and IL-1α, IL-6, IL-8, TNFα from were detectedmeasured in supernatants, all with ELISA at different time points after exposure. **Results:** The phosphorylation level of H2AX in DPSCs increaseds in a Dose dose and time-dependent manner immediately after the exposure then reacheds base line during the following 24 hours. increase in the phosphorylation level of H2AX was registered following CBCT exposure of the DPSCs. For MRE11 response was weaker and only detected for the modifications were milder, observed only for the highest IR dose. PNotably, pPro-inflammatory CKs showed significant and persistent increases according to time and IR dose. **Conclusions:** The eExposures of DPSCs generatedto IR to during CBCT of DPSCs did not determine induct persistent and severe modifications of the levels of DNA damage molecules expression, however significant and persistent increases in the amounts of the tested pro-inflammatory CKs were observed until 6 h after exposure.

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Commented [BN10]: Isn't their origin the exfoliated deciduous tooth? I think the flow cytometry is used to assess their 'stem cell phenotype'.

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Introduction

Cone Beam Computed Tomography (CBCT) is a multipurpose radiographic tool for diagnosis, treatment planning and follow-up in dental ~~practice which~~practice, which provides three-dimensional information at a relatively high resolution with considerably lower radiation dose than conventional multislice Computed Tomography (CT)[1, 2]. CBCT was ~~also~~ introduced in dental pediatric radiology, ~~mainly~~ ~~notably~~such as in the field of orthodontics. A typical orthodontic patient is subjected to several conventional or two-dimensional radiographs: cephalometric, panoramic and intraoral.[3] Moreover, the number of CBCT examinations in children is constantly increasing in the past few years, therefore due to repetitive CBCT scans radiation doses might reach ~~significant~~ important levels. According to the radiation doses of dental radiographs described in the literature t~~T~~hese patients may receive an effective radiation dose of up to 477 mSv, which corresponds to 58 days of natural background radiation~~, according to the radiation doses of dental radiographs described in the literature~~[4, 5].

Ionizing radiation (IR) is a known carcinogen,~~,~~ its effects on human tissues depending on the absorbed dose.[6] The carcinogenic risk at low doses (below 100 mSv) is controversial and needs to be thoroughly investigated. Concerns about radiation exposure are greater for younger patients because they are at least three~~3~~ times more sensitive to radioinduced malignancies compared to adults[7, 8]. In the recent years, several studies evaluated the risk related to low-dose radiation

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Commented [B19]: Being a phrase with a general comparison in the Introduction section, I think more details can be provided by the cited articles. But we can add the epidemiology paper. Misa, can you add that article, please?

Commented [RJ20]: effective radiation dose up to 477 microsievert not millisievert!!!!

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exposure, most of them being epidemiological studies, for example a study of the Hiroshima and Nagasaki survivors[9] and another performed on occupational exposure to IR[10]. Some biomarkers described earlier can offer information about the individual's response to the environmental carcinogenic agents[11] or specifically to IR[12, 13].

Human mesenchymal stem cells (hMSCs) were defined by the International Society of Cellular Therapy based on the following criteria: hMSCs must be able to adhere to plastic surface under standard tissue culture conditions, must express certain markers, including CD73, CD90, and CD105, and lack the expression of other markers, such as CD45, CD34, CD14, CD79α or CD19 and HLA-DR surface molecules. The hMSCs are also capable of differentiating into osteoblasts, chondroblasts and adipocytes under appropriate *in vitro* conditions[14]. Research into hMSCs biology has been hampered in part because of a lack of unique definitive hMSC surface markers. Lower doses of IR exposures may result later in radiogenic cancers virtually in all locations in the body, but ~~these~~ the occurrence of these pathologies are probabilistic in nature. Therefore, given the paramount importance for human health and IR risk prevention strategies, the clarification of whether ~~the~~ hMSCs residing in organs/tissues are responsible for such outcomes becomes a major ~~issue~~ point for the scientists to address[15]. An alternative source of hMSCs are some of the human stem/progenitor cells, related to teeth, such as dental pulp stem cells (DPSCs).

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[9] Toshiteru Okubo; Long-term epidemiological studies of atomic bomb survivors in Hiroshima and Nagasaki: study populations, dosimetry and summary of health effects. Radiat Prot Dosimetry 2012; 151 (4): 671-673. doi: 10.1093/rpd/ncs179

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[15] Sokolov M, Neumann R, Lessons Learned about Human Stem Cell Responses to Ionizing Radiation Exposures: A Long Road Still Ahead of Us, Int. J. Mol. Sci. 2013, 14, 15695-15723; doi:10.3390/ijms140815695.

These cells are capable of differentiating in odontoblasts, osteoblasts, adipocytes even neuron-like cells and have shown to play a crucial role in tooth development and regeneration processes following dental trauma [16,17,18,19].

IR produces DNA damage directly by the ionization of the DNA backbone and indirectly by the ionization of water and production of free radicals which exert their damaging action on the DNA molecule, proteins and lipids. The response to this attack is the activation of signaling and effector pathways ~~aiming~~ aimed to repair DNA damage and restore the stability of the genome[20]. DNA damage caused by IR is represented by single (SSBs) or double stranded breaks (DSBs) of DNA, the latter being the principal lesion that, 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 subsequently result in malignant cell transformation[21].

Among the histone proteins (H2A, H2B, H3 and H4), H2AX is a variant of H2A family, representing ~ –10% of it, randomly incorporated into histones throughout the DNA[22]. The formation of DSBs is always followed by the phosphorylation of H2AX, usually on Ser 139 residue, the phosphorylated H2AX being termed as γH2AX[23]. PI3K pathway molecules, such as

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ataxia telangiectasia mutated (ATM), ATM-Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK) are responsible for this process[24].

The initial signal (phosphorylation) ~~as initial signal~~ once triggered, many repair proteins are attracted and bind to γH2AX. ~~Such repair may be found in complex is the~~ such as the MRN complex proteins, consisting of human meiotic recombination 11 protein (MRE11), RAD50 and defective gene responsible for the Nijmegen breakage syndrome (NBS1). This complex is implicated in the detection of DSBs and the recruitment of ATM to the site of the damage[25,26]. The attracted ATM ~~is targeting~~targets substrates such as breast cancer protein 1 (BRCA1), p53 binding protein 1 (53BP1) and 2 (53BP2) and the mediator of DNA damage checkpoint (MDC1), as well as the checkpoint proteins, Chk1 and Chk2[27]. These processes are directed to stop ~~the~~ progression of the cell cycle and to activate proteins responsible for DNA repair, thus promote genome stability. After DNA is repaired, γH2AX is dephosphorylated by PP2A phosphatase which regulates γH2AX levels in human cells[28]. or γH2AX can also be removed completely and replaced by a normal H2AX.

Cytokines (CKs), soluble "factors" secreted by various cell types after stimulation, have major relevance in radiation research[29,30]. Specifically, pro-inflammatory CKs as components of early response programs to irradiation, being rapidly activated after tissue ~~irradiation~~ exposure contribute to the immune system's modulation and increased expression of proto-oncogenes and other CKs[31]. The pro-inflammatory phase persists until the apparent challenges to the host integrity are eliminated. Antioxidant and anti-inflammatory CKs then act to restore homeostasis. CKs

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Radiat Res. 2012 Dec;178(6):505-23. doi: 10.1667/RR3031.1. Epub 2012 Oct 29. Review.

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production is time-dependent, peaking usually at 4-24 h after irradiation with subsequent decrease to baseline levels within ~~24 h to a~~ few days[32]. Factors ~~that~~ influencinge the CKs production are ~~the~~ radiation dose and ~~the~~ type of ~~the~~ tissue. Inflammatory reactions induced by irradiation are mediated most frequently by IL-1α, IL-6, IL-8, TNFα, within minutes to hours after an exogenous stress signal[33, Schaue et al., 2012].

The present study ~~aroused~~ rose from the necessity to evaluate the radiation risk in pediatric patients exposed to repeated CBCT examinations. The radiosensitivity of hMSCs was examined in ~~relatively~~ few studies, primarily focused on bone-marrow derived cells exposed to high doses of radiation[34],[35] warranting further research. 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. ~~, t~~The main aim of such studies ~~being~~is the identification and characterization of the role of DNA damage and its repair following clinically relevant irradiation schedules. To the best of our knowledge the biological changes in human DPSCs exposed to low-dose irradiation derived from CBCT exposure have not yet been investigated previously.

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Material and methods

Establishment of the ~~dental pulp stem cell~~DPSCs cultures

The culture of ~~DPSC~~ stem cells from human exfoliated deciduous teeth (SHED) was established as previously reported[36,37,38, Gronthos et al, 2000]. Briefly, after decontamination with povidone-iodine

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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 $10^4/cm^2$ and the cultures were maintained with Dulbecco's Modified Eagle Medium (DMEM) 1 g/L D-Glucose (Sigma-Aldrich, MO, USA), supplemented with 10% Fetal Bovine Serum (Sigma-Aldrich, MO, USA), 1% Penicillin/Streptomycin (Sigma-Aldrich, MO, USA), at 37°C with 5% CO_2. Cells were detached by trypsinization at 70–80% confluence and either replated at the same density or frozen at -80 C°. For all experiments, cells were used between passages 2 and 4. Their phenotype was ~~established~~ confirmed by immunocytochemistry staining and flow ~~-~~cytometry analysis.

Immunocytochemistry staining

The cells were seeded at a ~~cell population~~ density of $3x10^4$ cells in 200 µl medium/well in 96 well plates. Upon reaching subconfluency, the cells were washed 3 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 ~~at room temperature~~, followed by blocking of unspecific antibody bound with 10% Bovine Serum Albumin (BSA) in PBS, 20 minutes at room temperature. Staining was performed for the following stem cell markers: CD44 FITC (fluorescein isothiocyanate) (Becton Dickinson), dilution 1:20; CD73 PE (phycoerythrine), SSEA-4 (stage-specific embryonic antigen-4), ALP (alkaline phosphatase), Sox-2 (SRY (sex determining region Y)-box 2) and Oct3/4 (octamer-binding transcription factor 4) (Santa Cruz Biotechnologies), 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 ~~in dark~~ with secondary antibody goat anti-mouse IgG ~~labelled~~labeled with FITC (Santa Cruz Biotechnologies) (1:50). After each step that the cells were washed with PBS~~Each step was followed by three washes with PBS~~. For nuclei staining the slides were mounted with 4,6-diamidino-2-phenylindole (DAPI) ~~mounting medium~~ (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.

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Flow-cytometry analysis

For each sample, $10x10^5$ 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 washes with cold PBS. Stained cells were analyzed with a FACS Canto II, 6-color flow-cytometer (Becton Dickinson) using FACS Diva version 6.1.3 software (Becton Dickinson).

Cultivation and irradiation of the ~~stem cells~~DPSCs

Cells harvested at the cell population density of $15x10^3$/well were incubated 24 h in cell culture conditions described above. Some plates were considered 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 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 at 0, 0.5 h and 24 h after CBCT irradiation and maintained in this solution at 4°C until processing.

Irradiation of the stem cells with CBCT

A cylindrical polymethyl methacrylate (PMMA) phantom (23x16 cm), corresponding to a 10 years old child's head was used for the irradiation of the cells (Fig.1.a). 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 (Fig.1.b). The Planmeca 3D ProMax (Planmeca, Finland) CBCT device was used for irradiation (Fig.1.c). The skull scanning protocol was: ~~vol.~~fField of view 230x260mm, 96kV, 9mA, 27s, DAP 3462 mGy*cm^2, ~~CTDI 3.7 vol*mGy,~~ voxel 600 µm. ~~The~~ Radiation doses were measured with Thermo Luminescent Dosimeters (TLDs) 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 (low jaw exposure or mandibular jaw exposure); 12.8~~3~~ mGy, corresponding to 1 skull exposure; 34.0~~3.95~~ mGy, corresponding to 3 consecutive skull exposures; 66.0~~5.99~~ mGy, corresponding to 5 consecutive skull exposures and 107.7~~3~~ mGy, corresponding to 8 consecutive skull exposures. For the determination of H2AX and MRE11 proteins all the doses mentioned above were tested. The inflammatory CKs production was evaluated after 1 skull exposure, the most frequently used

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procedure in the clinical practice, which corresponds to 12,83 mGy. Aiming to obtain significant results a higher CBCT dose was also tested: 107,73 mGy.

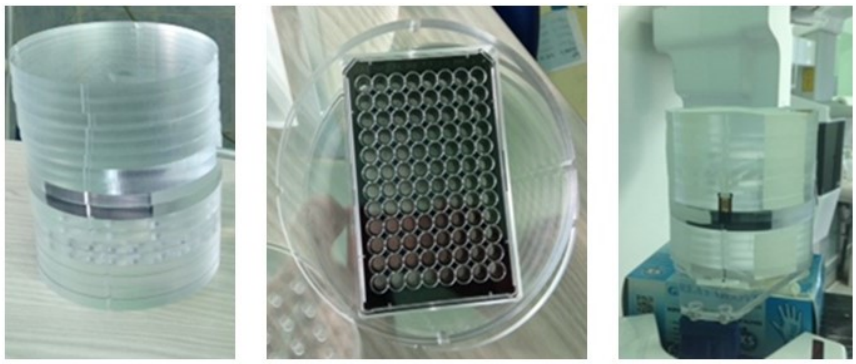

Fig.1. Polymethyl methacrylate (PMMA) phantom used for the irradiation of the cells (a). The 96 well plate containing the stem cells at the height corresponding to the location of the oral cavity (b). The phantom with the 96-well plate placed in Planmeca 3D ProMax CBCT device used for irradiation (c).

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

For the determination of histone H2AX protein a cell-based ELISA assay with fluorogenic substrate was used to measure total (H2AX) and phosphorylated (γH2AX) proteins in whole cells (R&D Systems, Abingdon, UK). The method was done 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 determine the proteins according to their phosphorylation status. Measurements were performed with the BIOTEK Synergy 2 microplate reader (Winooski, USA) operated in fluorescence mode, at 600 nm for the phosphorylated H2AX and at 450 nm for the total H2AX. —γH2AX/total H2AX fluorescence ratios were calculated, in accordance to manufacturer's recommendation.

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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, USA). 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, USA) operated in absorbance mode, at 450 nm wavelength.

Inflammatory CKs (IL-1α, IL-6, IL-8, and TNFα) 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 ~~of the data~~ was done using GraphPadPrism software program, version 5.0 (GraphPad, San Diego, CA, USA). Statistical comparisons between groups were made by U~~u~~~~U~~npaired t~~- Test~~ test ($p<0.05$ statistical significance).

Results

Immunocytochemistry ~~staining~~& flow-cytometry analysis confirm the stemness of the ~~dental pulp cells~~DPSCs

~~IFirst at all, we aimed to characterize the cells used in the following experiments. As expected,~~ i~~I~~mmunocytochemistry demonstrated a pluripotent stem cell phenotype, with both mesenchymal and embryonic characteristics. T~~Indeed, t~~~~T~~he 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 admitted mesenchymal stem cells markers (CD44, CD73, Oct3/4 and ALP) (Fig.2~~1~~.).

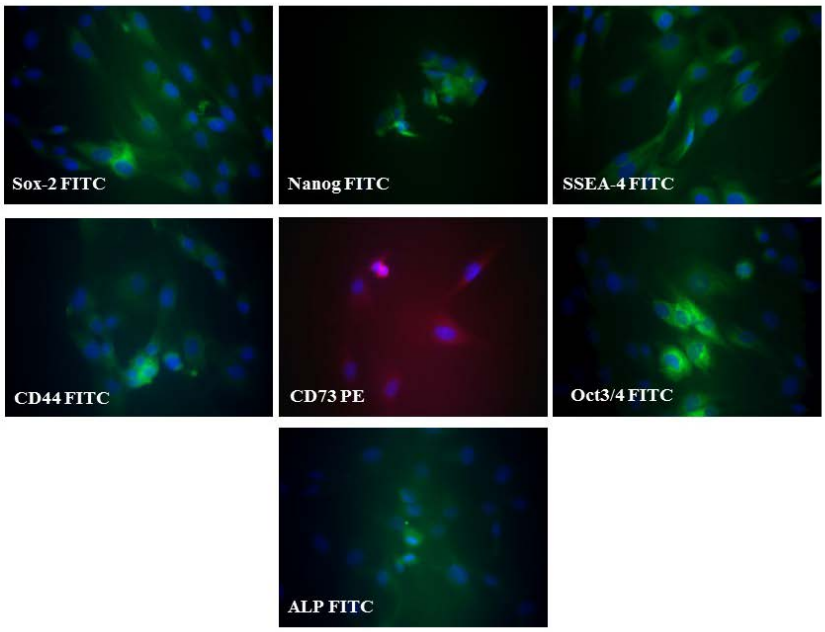

Fig.2~~1~~. Immunocytochemical staining for embryonic stem cells pluripotency (Sox-2, Nanog and SSEA-4) and mesenchymal stem cells (CD44, CD73, Oct3/4 and ALP) markers. Cells were labeled with fluorescein isothiocyanate (FITC), ~~ or~~ phycoerythrine (PE) or 4',6-diamidino-2-phenylindole (DAPI).

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As well, ~~Flow-cytometry analyses~~

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f~~F~~low-cytometry results showed intense ~~positivity~~ expression (88,9-99,9%) for mesenchymal stem cells markers: CD44, CD166, CD73, CD49e, CD29 and CD105, whereas cells were negative (0,1-0,2%) for CD34, CD45 and CD117 markers (Fig.3~~2~~.).

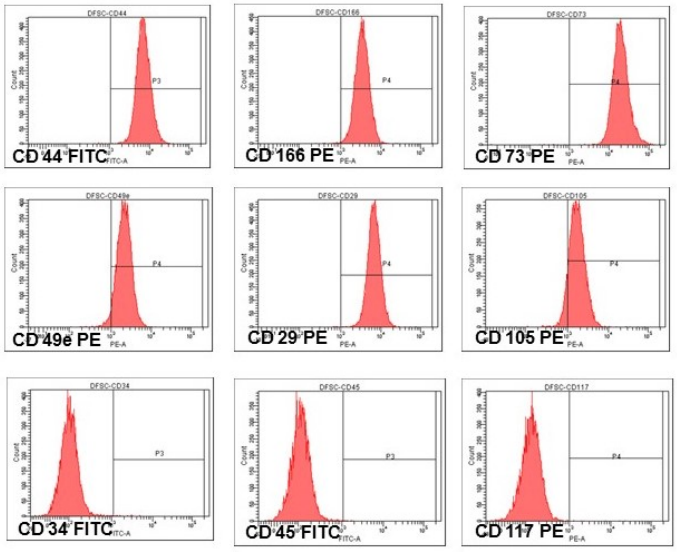

Fig.~~32~~. Flow-cytometry histograms of cells stained with monoclonal antibodies characteristic for mesenchymal stem cells. Cells were labeled with fluorescein isothiocyanate (FITC) or phycoerythrine (PE).

CBCT exposures induct a dose dependent γH2AX response in DPSCs~~dental pulp stem cells~~

~~*Assessment of histone H2AX proteins in DPSCs after CBCT irradiation*~~

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The calculated γH2AX/total H2AX fluorescence ratios showed that in the used dose range (0-107.73 mGy) the exposure of the cells to CBCT induced modifications in the phosphorylation level of the assessed protein (Fig.~~43~~.). These changes appeared immediately after CBCT exposure and culminated after 0.5 h. Differences were dose-dependent, consequently the most significant modifications were registered for doses corresponding to 5 and 8 skull exposures ($p<0.001$) immediately after CBCT exposure and for doses corresponding to 3, 5 and 8 skull exposures ($p<0,001$) at 0,5 –h, respectively. Hence, the most marked modification in the H2AX phosphorylation level occurred at 0.5 –h, the differences becoming insignificant 24 h after exposure.

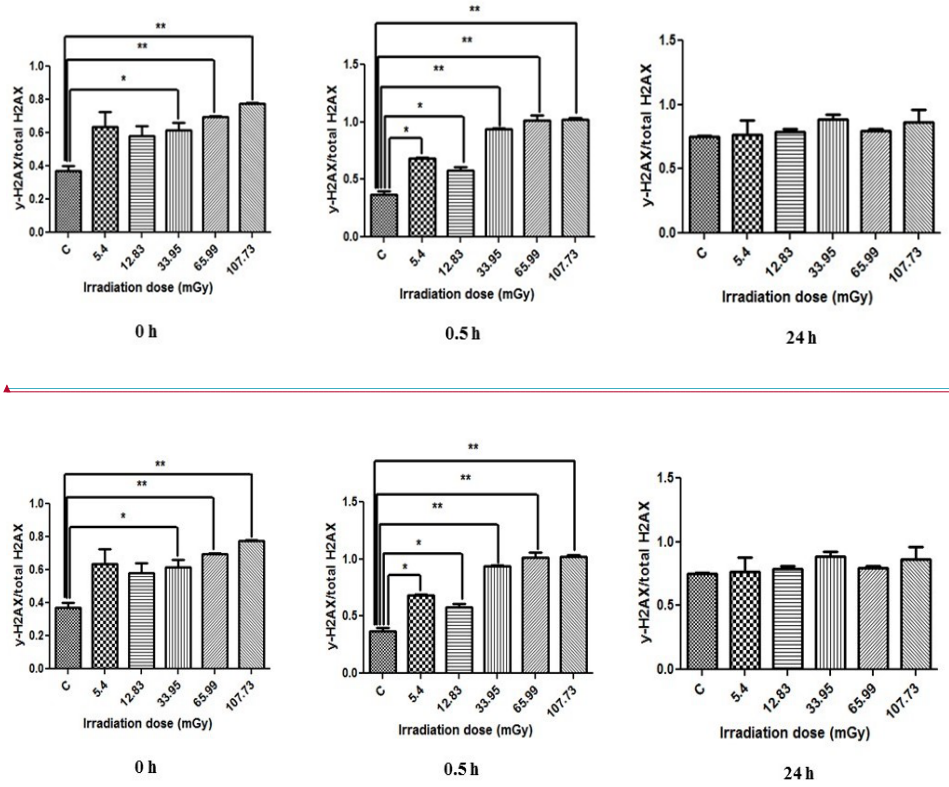

Fig.4. γH2AX/total H2AX fluorescence ratios in DPCSs after CBCT irradiation. Values of H2AX proteins are expressed as relative fluorescence units. Statistical comparisons between groups were made by Unpaired t test, *p<0.05; **p<0.001; (Means ± SEM, n=3).

~~Fig.3. γH2AX/total H2AX fluorescence ratios in DPCSs after CBCT irradiation. 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; (Means ± SEM, n=3).~~

CBCT exposures induct a weak expression of MRE11 in ~~dental pulp stem cells~~DPSCs

~~*Expression of MRE11 protein after CBCT irradiation of the DPCSs*~~

MRE11 protein was slightly affected by CBCT irradiation and only after the exposure to the highest dose (107.73 mGy) ~~at~~ 0.5 h ~~from~~ after irradiation ($p<0.001$) (Fig.54.). For other doses and time points of evaluation~~;~~ there wasere no ~~important~~ relevant ~~modifications~~ change in the relative amount of this protein.

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Commented [MM33]: For all pictures in the manuscript: Is it needed to add figure A, B, C, …?

Commented [B34]: In my opinion it is not necessary, given that the time points (0; 0.5; 24 h) are specified bellow. So we are referring to time points and not to letters in the text.

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Commented [B35]: You have recommended to use one-way Anova for the evaluation of the statistical significance. I tried to do it, but at a second thought, our goal in this paper is to see the effects caused by different doses of irradiation as compared to control. However, Anova is evaluating the statistical significance in the whole group. Of course, Dunett post-test can compare separately each group to the control. Interestingly, with this test we can obtain even higher significance for H2AX, but we do not obtain significance for MRE11 at all!!! So, I don't think it is good. And the reason why I choose Unpaired t test is that we had different number of determinations in control group vs irradiated groups. Besides, the groups differ among each other, since not the same cells were evaluated before and after irradiation with several doses (we had 6 groups of cells: the control and 5 groups irradiated with different doses).

Commented [BN36]: I'm not sure, but is it not better to do a One-way ANOVA and use post-tests to determine the correct significance levels?

Commented [B37]: I used Unpaired t test in order to compare every two groups (basically, every dose with the control group), because Paired t test can be done when we are referring to the same cells (before and after treatment), but here are 6 different groups (C + five treated with 5 different irradiation doses).
One-way Anova makes a global evaluation of the significance between all the groups, but if we do Dunett post-test, we can compare every group with the control. So, it is also correct, I think.
I replaced the figure, noting the significances calculated with Anova/Dunett tests. As you see, we obtained even more significance.

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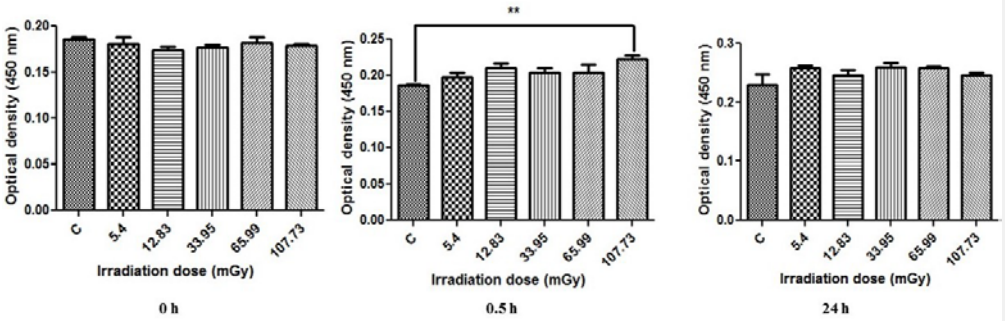

Fig.54. Expression of MRE11 protein after CBCT irradiation in DPSCs. Statistical comparisons between groups were made by Unpaired t test, **p<0.001; (Means ± SEM, n=3).

CBCT exposures induct inflammation in ~~dental pulp stem cells~~DPSCs

~~*The production of inflammatory CKs after CBCT irradiation of the DPSCs*~~

IL-1α, IL-8 and TNFα expressions were strongly ~~positively~~ influenced by the CBCT exposure, generally in a dose and time dependent manner (Fig.65.). ~~Thus,~~ IL-1α increased significantly at 6 h after CBCT exposure for both doses ($p<0.001$ and $p<0.0001$, respectively); after 24 h this molecule remained ~~evaluated~~ elevated at a significant level ~~as~~ when compared to control ($p<0.0001$, for both doses). However, IL-8 ~~molecule suffered~~level ~~slighter modifications, being~~was significantly up-regulated at 6 h only by the higher CBCT-derived dose (107.73 mGy, $p<0.001$), and remain~~ed~~ing slightly up-regulated by both irradiation doses ($p<0.05$). The level of TNFα showed strong modifications for both CBCT-derived doses and for both time points tested in the present study ($p<0.0001$, for all parameters). Although values remained significantly up-regulated after 24 h for all of the three CKs assessed (IL-1α, IL-8 and TNFα), their absolute values ~~showed~~ trend to decrease ~~decreasing tendency in~~over time~~, therefore evaluations at later time points have not been performed~~. IL-6 ~~molecule~~ level was not ~~induced~~ significantly affected by the tested CBCT doses at the selected time points. The only notable difference was recorded between the un-irradiated controls, at 24 h after irradiation, probably as a result of cell multiplication.

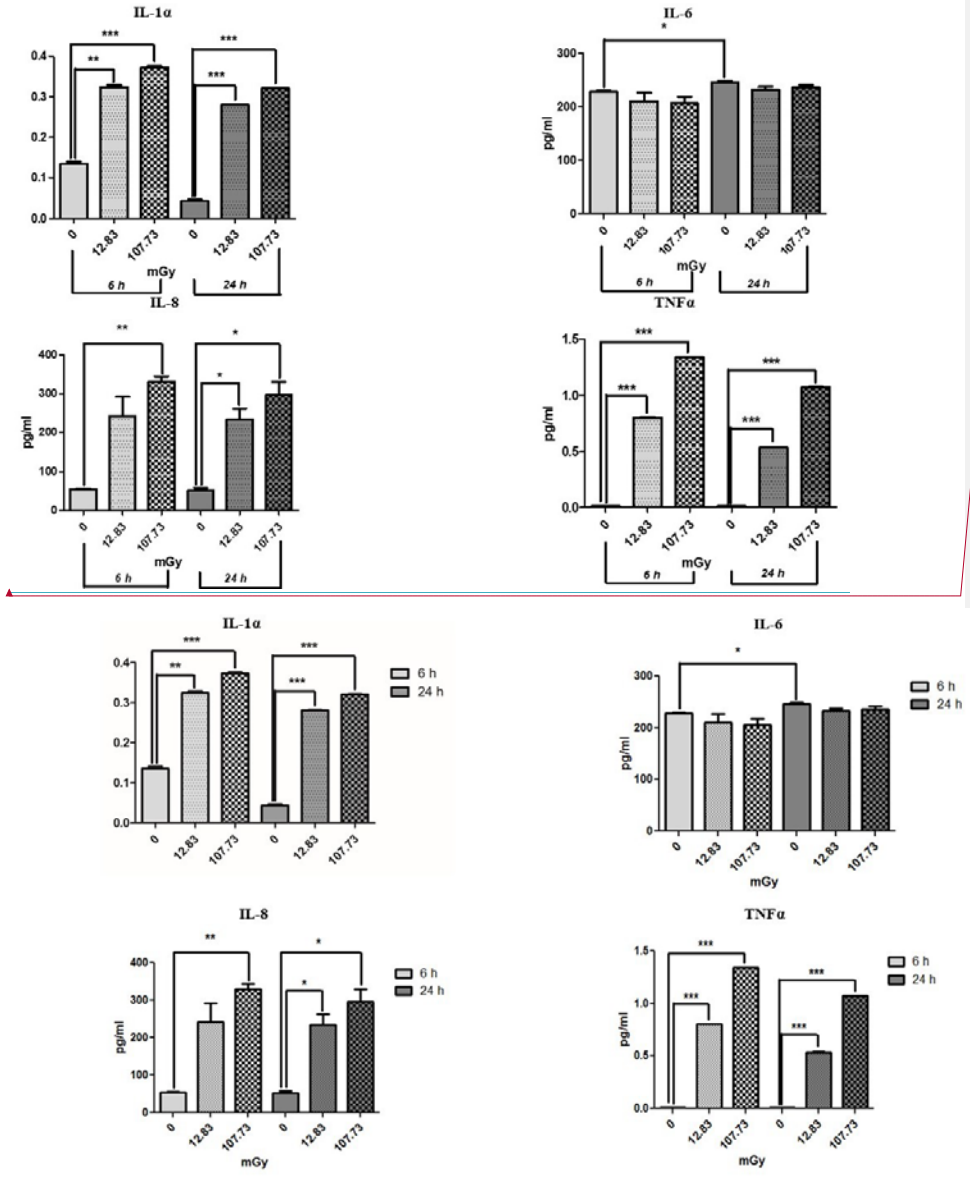

Fig.65. Inflammatory CKs' production after CBCT irradiation of the DPSCs. Statistical comparisons between groups were made by Unpaired t test, *p<0.05; **p<0.001; ***p<0.0001 (Means ± SEM, n=3).

Discussions

Cone Beam Computed Tomography (CBCT) has several applications in dentistry due to the provided high-resolution three-dimensional informations, but also represent a potential risk of exposition to IR, especially for pediatric population considering the repetitive CBCT scans over time. The IR doses derived from CBCT examinations are lower than those resultinged from conventional multislice CT procedures but remain higher than those resulted fromdelivered from conventional dental X-ray imaging. Even though CBCT is considered to be "low dose" imaging, as defined by the High Level Expert Group (HLEG)[39], the with doses ranging may vary from between a few μSv to mSv/examination. Of major importance is to identify and to quantify the precise effects and potential risks of clinically relevant IR doses, in the exposed tissues, especially in stem cells originated from teeth, which are directly irradiated during CBCT examination.

Earlier studies evaluated the effects of low-doses of IR in atomic bomb-survivors Okubo, 2012 and occupationally exposed population[40, Richardson et al., 2015], while similar studies on stem cells endorsed the effects of high IR doses on bone-marrow derived stem cells Yeh et al., 2012; Mussano et al., 2010. One study assessed the effects of orthodontic radiography-derived X-rays in children measuring the number of micronucleated cells in the exfoliated oral muccosa cells. They found a certain amount ofdetected the DNA damage but it was lower than the threshold value for carcinogenesis. On the other hand, this study found this procedure to be cytotoxic to buccal mucosa exfoliated cells.[41]. Another interrelated study evaluated the DNA damage induced by X-rays with micronucleus assay as an endpoint to study the bystander effect of X-rays on bone marrow mesenchymal stem cells and demonstrated observed increased micronuclei formation in bone marrow mesenchymal stem cells [42].

[39] www.hleg.de

[40] Thierens H, Vral A, Morthier R, Aousalah B, De Ridder L. Cytogenetic monitoring of hospital workers occupationally exposed to ionizing radiation using the micronucleus centromere assay. Mutagenesis. 2000 May;15(3):245-9.

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[42] Chinnadurai M, Chidambaram S, Ganesan V, Baraneedharan U, Sundaram L, Paul SF, Venkatachalam P. Bleomycin, neocarzinostatin and ionising radiation-induced bystander effects in normal diploid human lung fibroblasts, bone marrow mesenchymal stem cells, lung adenocarcinoma cells and peripheral blood lymphocytes. Int J Radiat Biol. 2011 Jul;87(7):673-82. doi: 10.3109/09553002.2010.549536. Epub 2011 May 23.

The present study assessed the DNA damages and the induction of inflammatory CKs in DPSCs following the exposure to low-dose IR derived from CBCT. Due to the early appearance of γH2AX at DNA damage sites, this molecule is considered as a sensitive biomarker for IR-induced DSBs[43]. The phosphorylation of H2AX mediated by PIKK family proteins[44] in response to DSBs ~~is mediated by PIKK family proteins (ATM, ATR and DNA-PKs)[45]. The formed molecules~~ attract ~~other signaling and repair factors, among which the~~ MRE11 protein ~~, part of the MRN complex,~~ forming radiation-induced foci[46] with major role in ~~the~~ post-irradiation DNA repair processes. On the other hand, CKs produced following IR exposure mediate and regulate several cellular processes, especially at the site of the irradiation, such as apoptosis, protein synthesis, cell growth and inflammatory responses [Pernot et al, 2012]. Moreover, there are evidences that these pathways may impact upon DNA damage/repair responses through effects on PIKK proteins[47].

In the present study DPSCs were subjected to a low dose range of X-rays~~,~~ and the phosphorylation level of the histone protein H2AX ~~phosphorylation level~~ following irradiation showed dose and time dependent increase. The peak level of γH2AX was registered for the highest irradiation dose (107,73 mGy) after 0.5 h from CBCT exposure. This exposition (resulted upon 8 consecutive CBCT exposures) ~~is~~ corresponds ~~with~~to ~~a relatively high dose~~the admitted upper limit of low-doses ~~(resulted upon 8 consecutive CBCT exposures)~~, which is not ~~used effectively~~realistically used in the clinical practice for diagnostic proposes. However, the IR dose corresponding to ~~1~~ a full skull exposure, resulting from the most frequently used CBCT procedure caused significant H2AX phosphorylation, as well. It should be noted that t~~T~~hese ~~modifications~~ overexpressions were transient, ceasing after 24 h from exposure.

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[43] Valdiglesias V, Giunta S, Fenech M, Neri M, Bonassi S. γH2AX as a marker of DNA double strand breaks and genomic instability in human population studies. Mutat Res. 2013 Jul-Sep;753(1):24-40. doi: 10.1016/j.mrrev.2013.02.001. Epub 2013 Feb 13. Review.

[44] Wang H, Wang M, Wang H, Böcker W, Iliakis G. Complex H2AX phosphorylation patterns by multiple kinases including ATM and DNA-PK in human cells exposed to ionizing radiation and treated with kinase inhibitors. J Cell Physiol. 2005 Feb;202(2):492-502.

[45] ~~Wang H, Wang M, Wang H, Böcker W, Iliakis G. Complex H2AX phosphorylation patterns by multiple kinases including ATM and DNA-PK in human cells exposed to ionizing radiation and treated with kinase inhibitors. J Cell Physiol. 2005 Feb;202(2):492-502.~~

[46] Paull TT, Rogakou EP, Yamazaki V, Kirchgessner CU, Gellert M, Bonner WM. A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. Curr Biol. 2000 Jul 27-Aug 10;10(15):886-95.

[47] Kirshner J1, Jobling MF, Pajares MJ, Ravani SA, Glick AB, Lavin MJ, Koslov S, Shiloh Y, Barcellos-Hoff MH. Inhibition of transforming growth factor-beta1 signaling attenuates ataxia telangiectasia mutated activity in response to genotoxic stress. Cancer Res. 2006 Nov 15;66(22):10861-9. Epub 2006 Nov 6.

Similar results were obtained by Tu et al[48] in HeLa cells exposed to 1 Gy IR dose: the peak number of γH2AX foci was observed at 0.5 h after irradiation and gradually decreased afterward. For a higher dose (4 Gy), the number of γH2AX foci reached the maximum level after 1 h, with a second peak at 8 h from exposure. The same authors observed complete DSBs repair ~~at~~ after 8 h post-irradiation. The formation of γH2AX foci ~~within 1-3 min after irradiation, an increase in their number and~~ reaching a ~~plateau~~ maximum level after 10-30 min ~~were~~ was observed by other authors, as well[49,50]. Several studies found increased levels of γH2AX foci at doses ~~as low as~~ 1-10 mGy and decrease in their level up to 24 h in human fibroblast cultures[51, Mariotti et al., 2013], in mononuclear cells from patients exposed to X-rays derived from CT[52], in pediatric patients undergoing cardiac catherization[53] and in lymphocytes after coronary CT angiography[54]. Johnston et al[55] registered a linear proportionality between the received radiation dose (dose range 0-5 Gy) and phosphorylation level of H2AX in cell lysates and in *ex vivo* irradiated human whole-blood cells. The measurements in their study were done with a simplified enzyme-linked immunosorbent assay (ELISA), the response being quantified per samples instead of per cell basis, similarly to the present study. The H2AX phosphorylation signal was the highest at 6 h following 3 Gy radiation exposure of mice, the dissimilarity to our results being attributable to the different approach (*in vivo* vs *ex vivo*) and to the higher IR dose used in the cited study.

[48] Tu WZ, Li B, Huang B, Wang Y, Liu XD, Guan H, Zhang SM, Tang Y, Rang WQ, Zhou PK. γH2AX foci formation in the absence of DNA damage: mitotic H2AX phosphorylation is mediated by the DNA-PKcs/CHK2 pathway. .FEBS Lett. 2013 Nov 1;587(21):3437-43. doi: 10.1016/j.febslet.2013.08.028. Epub 2013 Sep 8.

[49] Rogakou EP1, Boon C, Redon C, Bonner WM. Megabase chromatin domains involved in DNA double-strand breaks in vivo. J Cell Biol. 1999 Sep 6;146(5):905-16.

[50] Sedelnikova OA, Rogakou EP, Panyutin IG, Bonner WM. Quantitative detection of (125)IdU-induced DNA double-strand breaks with gamma-H2AX antibody. Radiat Res. 2002 Oct;158(4):486-92.

[51] Rothkamm K, Löbrich M.Evidence for a lack of DNA double-strand break repair in human cells exposed to very low x-ray doses. Proc Natl Acad Sci U S A. 2003 Apr 29;100(9):5057-62. Epub 2003 Apr 4.

[52] Löbrich M1, Rief N, Kühne M, Heckmann M, Fleckenstein J, Rübe C, Uder M. In vivo formation and repair of DNA double-strand breaks after computed tomography examinations. Proc Natl Acad Sci U S A. 2005 Jun 21;102(25):8984-9. Epub 2005 Jun 13.

[53] Beels L, Bacher K, De Wolf D, Werbrouck J, Thierens H. gamma-H2AX foci as a biomarker for patient X-ray exposure in pediatric cardiac catheterization: are we underestimating radiation risks? Circulation. 2009 Nov 10;120(19):1903-9. doi: 10.1161/CIRCULATIONAHA.109.880385. Epub 2009 Oct 26.

[54] Kuefner MA, Grudzenski S, Hamann J, Achenbach S, Lell M, Anders K, Schwab SA, Häberle L, Löbrich M, Uder M. Effect of CT scan protocols on x-ray-induced DNA double-strand breaks in blood lymphocytes of patients undergoing coronary CT angiography. Eur Radiol. 2010 Dec;20(12):2917-24. doi: 10.1007/s00330-010-1873-9. Epub 2010 Jul 13.

[55] Johnston ML, Young EF, Shepard KL. Whole-blood immunoassay for γH2AX as a radiation biodosimetry assay with minimal sample preparation. Radiat Environ Biophys. 2015 Aug;54(3):365-72. doi: 10.1007/s00411-015-0595-4. Epub 2015 May 3.

Therefore, the present findings in accordance to most of the literature data sustain the maximum H2AX phosphorylation level at 0.5 h and decrease to baseline after 24 h from exposure to low doses of various cell types.

The generation of DSBs triggers the relocalization of many DNA damage response proteins, such as MRE11/NBS1/RAD50, MDC1, 53BP1 and BRCA1[Paull et al., 2000] to nuclear foci where these proteins colocalize and interact with γH2AX. Therefore, the latter act as an "anchor" that facilitates the assembly of multiple DNA-protein interaction, thus preventing the error-prone repair of DSBs[56]. On the other hand, Yuan and Chen[2009] proposed that the MRN complex ~~can~~could recognize the formed DSBs alone, as well, independently to the H2AX-mediated DNA damage signaling cascade. This protein apparently works together with CtIP, a DNA damage response protein which promotes the resection of DNA DSBs essential for meiotic recombination and regulate DNA damage checkpoints[57].

MRE11 protein having the ~~capability~~ capacity to recognize the DNA DSBs via the formed and attached γH2AX foci, showed ~~milder~~slighter modifications upon irradiation than H2AX in the present study. Changes were obvious ~~at 0.5 h after irradiation and~~ only for the highest dose (107.7~~3~~ mGy) ~~selected for testing~~at 0.5 h after irradiation. Later, at 24 h post-irradiation, these modifications disappeared. ~~-~~Colin et al[58] observed a non-linear dose-dependent increases of the severity of DSBs and of the MRE11 foci formation in three cell lines with different degrees of radiosensitivity after irradiation of the cells with higher (up to 2 Gy) IR doses, which is consistent to our results.

CKs production following IR exposure can initiate a range of cellular processes including apoptosis, protein synthesis, cell growth and inflammatory responses with 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~~ then 24 h~~, in a time frame~~in other words, when the peak secretions of CKs are expected according to literature

[56] Bassing CH, Chua KF, Sekiguchi J, Suh H, Whitlow SR, Fleming JC, Monroe BC, Ciccone DN, Yan C, Vlasakova K, Livingston DM, Ferguson DO, Scully R, Alt FW. Increased ionizing radiation sensitivity and genomic instability in the absence of histone H2AX. Proc Natl Acad Sci U S A. 2002 Jun 11;99(12):8173-8. Epub 2002 May 28.
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data[59,60]. One full skull exposure (corresponding to 12,83 mGy), t~~T~~he most frequently used procedure according to the CBCT clinical protocols ~~The CBCT clinical protocol delivering the highest dose~~~~The most frequently used CBCT procedure, one full skull exposure (corresponding to 12,83 mGy),~~ was selected for testing and also repeated to reproduce a higher dose (107,73 mGy) ~~aiming~~ in order to obtain significant results. IR-induced modifications were registered for IL-1α and TNFα, both CKs showing significant increases at 6 h, even after the exposure of the cells to the smaller dose (12,83 mGy). Nevertheless, b~~B~~oth CKs, tested at 24 h after the exposures to both IR doses, ~~showed decreasing tendencies in their absolute values~~trend to reach the base line. Overall, equivalent~~Similar~~ findings were obtained for IL-8. However, ~~, except that at the earlier time point (6 h), only the highest dose caused significant difference, while after 24 h similar decreasing tendency was observed in its absolute value. For~~regarding IL-6 the tested IR doses did not cause any ~~significant~~ changes at the ~~selected~~ studied time points.

Similar to these results, elevated levels of IL-1α and TNFα have been found after irradiation of various human or mammalian cells by others, as well[61,62,63]. ~~I~~It has been demonstrated that the secretion of CKs is cell ~~line~~ type specific and that the pro-inflammatory CKs (IL-1α, IL-6, IL-8 and TNFα) are highly represented in irradiated conditioned media of the cells rather than immunomodulatory CKs (IFNγ, IL-2, IL-3 and IL-10)[64]. Others[65,66,67] also described a dose-dependent manner of CKs increase after irradiation, similarly to our findings. In the blood

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cells of Hiroshima[68] and Chernobyl[69] survivors, chronically exposed to IR, transcriptional modulations of several CKs were measured, including IL-2, ~~and inflammatory CKs and~~ TNFα/β, IL-1β, IL-2, IL-8, IL-10, IL-12β and macrophage colony-stimulating factor (M-CSF~~), respectively~~). In another study, IL-6 and IL-8 were identified in the growth medium of human dermal fibroblasts after exposure to 2 Gy, implicated in the establishment of bystander effects, as well[70]. ~~In this study~~ IL-6 was also reported to promote γH2AX foci formation, contrary to our findings, probably due to the higher dose used in the cited study as compared to ~~our~~the present work~~'s~~. TNF-α was shown to contribute to the radiation-induced DNA damage, including γH2AX foci formation that may occur ~~late~~ delayed after exposure, therefore it was associated with genomic instability[71]. The CKs tested in the present study were shown to alter the intrinsic radiosensitivity of the cells by linking DNA damage with cell fate decisions, including DNA repair, genomic instability, cell proliferation, differentiation and death[72]. Whilst DNA damage repair mechanisms represent an important aspect in responses to high doses of IR, subtle cellular responses such as CKs regulation and changes in the transcriptional level of these molecules may be also relevant following exposures to low doses of radiation[73].

Conclusion

The ~~tested~~ range of IR doses result~~inged~~ from CBCT exposure~~s~~ did not ~~determine~~ lead to severe and persistent ~~modifications~~ change of the levels of DNA damage ~~molecules~~ markers (γH2AX, MRE11) in DPSCs. Conversely, ~~we found~~ significant increases in the amounts of some

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pro-inflammatory CKs (IL-1α, IL-8 and TNFα) were found immediately after a standard clinical CBCT ~~exposure~~protocol, ~~which~~ and this expression ~~persisted~~remaineds ~~elevated~~ ~~in time~~24 h after the exposure. These findings r~~ise~~aised the question whether these molecules could influence the response to IR, by ~~inducing~~ promoting the inflammation response in the irradiated tissues. Our results encourage further studies on deciphering the CKs pathways in order to better understand and ~~circumvent~~ manage ~~the CBCT's~~ potential risks of CBCT radiation in pediatric patients.