



Research article

Application of the buccal micronucleus cytome assay on child population exposed to sinus X-ray

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ABSTRACT

Purpose: Diagnostic X-ray examinations of paranasal sinuses use a low-dose ionizing radiation to achieve medically indicated purposes. The effects of low-dose radiation are still controversial, making it a highly prioritized field of research. As there is a need to evaluate the effects of low-dose ionizing radiation and that children might be a more vulnerable population, we performed simultaneous physical dosimetry and buccal cell micronucleus cytome assay on pediatric patients before and after an X-ray examination of the sinuses.

Methods: The study comprised 20 subjects aged 11.9 ± 3.6 years, and $BMI < 25 \text{ kg/m}^2$. Physical dosimetry was performed using radiophotoluminescent (RPL) glass dosimeters placed on four positions on the head. The buccal cell micronucleus cytome assay was performed before and 14 ± 1 days after the X-ray exam, to monitor DNA damaging, replicative, cytostatic, and cell death effects.

Results: The doses in the primary beam ranged 371–1106 μGy and were several fold higher than at the other positions on the head. As for biological changes, we did not observe any DNA damaging effects. However, a significant increase in cells with condensed chromatin was observed, indicating more cells undergoing early stages of apoptosis. We also observed inter-individual differences between the subjects. A correlation between the doses detected and biological effects was not observed.

Conclusion: Although we did not observe significant increase in DNA damage, further studies are needed to increase the statistical power of the results and ensure patients' safety and optimal health care.

1. Introduction

Ionizing radiation is a known carcinogen capable of inducing DNA strand breaks and the formation of reactive oxygen species (ROS), which indirectly may also lead to the formation of strand-breaks [1,2]. On the other hand, ionizing radiation is the basis for many therapeutic and diagnostic procedures. If appropriately prescribed and performed, such procedures deliver low doses to patients, thus minimizing unwanted risks and assuring many health benefits [3]. The number of annual diagnostic medical examinations worldwide is estimated at 3.6 billion, of which 350 million are performed on children under the age of 15 [4]. Although in the general population only 3.2 % of X-ray procedures are performed on the skull, this ratio is much higher for children, where the head/skull is the most examined region of the body with up to 19 % of all examinations [3]. The investigation of effects in

children is of high importance as they represent a specific vulnerable population that might be between 2- and 3-fold more sensitive to certain health effects [4]. Additionally, they have a longer life expectancy, therefore enabling a longer latency period to express radiation-related effects. The International Commission on Radiological Protection (ICRP) also highlights the importance and recommends assessing radiation-related risks for the pediatric population [5].

As there is no "safe dose", there remains a constant need to develop more sensitive and reliable biomarkers of effect as well as a need to monitor early molecular effects that might help us determine the stochastic effects of irradiation. Melodi (Multidisciplinary European Low Dose Initiative) (<http://www.melodi-online.eu/>) has also set priority research to identify and validate novel biomarkers of health effect related to cancer and evaluate cancer risks in biological systems on epidemiological data [6].

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The micronucleus (MN) assay on peripheral blood lymphocytes is one of the best-validated cytogenetic methods to monitor chromosome/chromatid damage in exposed human populations, and micronucleus frequency is considered a reliable cancer predictive biomarker [7–9]. Its accuracy and usefulness as a tool for biodosimetry in potential large-scale emergencies was determined by the Realizing the European Network of Biodosimetry (RENEB) study [10]. As the method requires blood sampling, there was room to minimize its invasiveness, so the MN assay was adopted to be performed on buccal epithelial cells. Besides being less invasive, buccal MN frequency correlates well with lymphocyte MN frequency that indicates its sensitivity and applicability in biomonitoring [11–15]. Based on cell and nuclei appearance, buccal MN cyto (cyt) assay can determine several cytotoxic and genotoxic effects of chemical and physical agents, including ionizing radiation.

The aim of the present study was to investigate the effects of diagnostic paranasal sinus X-rays on buccal epithelia cells in the pediatric population. As for biomarkers of effect, we decided to use all parameters of the buccal MN cyt assay and evaluate them before the sinus X-ray exam, and 14 days after the exam. Special attention was given to correlate the selected biomarkers to physical dose values in particular head regions. For this purpose, the RPL dosimetry system was used because it was successfully applied in our previous studies [16–18].

2. Subjects and methods

2.1. Study population and sampling

The Hospital and Institute's Ethics committees approved the preliminary study wherein 20 pediatric volunteers were recruited with the consent of their parents or legal guardians. The inclusion criteria were age < 18 years, body mass index (BMI) < 25 kg/m², last exposure to diagnostic irradiation at least 1 month ago, residence in the Zagreb region, and valid indication for a sinus X-ray exam. Detailed population characteristics are shown in Table 1. After filling in an informed consent and questionnaire, the volunteers rinsed their mouths with water and then gently scraped both cheek mucosa using a toothbrush and placed cells into a conical tube containing cold buccal cell buffer (1.6 g/L Tris-HCl, 38.0 g/L EDTA and 1.2 g/L of sodium chloride, pH 7.0) before the X-ray exam. All samples were coded and stored at 4 °C for up to 4 h until slide preparation. To assess the possible radiation-induced cyto/genotoxic effects, the collection of exfoliated buccal epithelial cells was repeated 14 ± 1 days after the X-ray exam, as a middle of the follow up period recommended by Thomas et al. [12].

2.2. Physical dosimetry

After the buccal cell sampling, the radiophotoluminescent (RPL)

Table 1
Study population characteristics.

	Value
sex ratio (female: male)	11:9
age (years)	11.9 ± 3.6 [5 – 17]
height (m)	1.53 ± 0.18 [1.11–1.75]
mass (kg)	43.6 ± 13.3 [20–64]
body mass index (kg/m ²)	18.1 ± 2.4 [13.3–24.1]
use of mouthwash (%)	0.05
use of medications (%) [#]	45
family cancer history (%)	40

Data presented as means ± SD and range, ratio or percentage.

[#] mostly salbutamol and desloratadine, but not antibiotics or anticancer drugs.

glass dosimeters (the Dose Ace GD-352 M, AGC Techno Glass Co. Ltd., Japan) were positioned on the occiput to assess the dose at the entry of the primary beam, below the tongue to assess the dose in the buccal cavity, in front of thyroid, and on the left supraorbital foramen to assess doses at radiosensitive organs (thyroid and eye lens). The RPL dosimeters consist of a silver-activated phosphate glass cylinder, 1.5 × 12 mm. The glass cylinder is placed in a dosimeter holder containing a 0.75 mm thick tin (Sn) energy compensation filter. Each dosimeter has a unique identification number. After irradiation, the color centers generated in the glass during UV excitation in the reader emit a radiation-induced orange fluorescent light. The light intensity is then measured (read-out). The initial and final reading of dosimeters was performed on a Dose Ace system instrument, FGD-1000SE (AGC Techno Glass Co. Ltd.). This fully automatic system (built-in calibration factors, reference glass for correction) has the advantage of quick and easy reading [16]. The dosimeter annealing and treatment were conducted according to the manufacturer's recommendations. The calibration, control/transport and working dosimeters were not mixed during the experiment. Every calibration irradiation was carried out in the Secondary Standard Dosimetry Laboratory of the Ruđer Bošković Institute with a dose of 5 mGy by ¹³⁷Cs source. Generally, the minimum detectable dose for the applied RPL dosimetric system is 1 μSv according to the producer. However, the detection threshold defined as three times the standard deviation of unirradiated dosimeters in our experimental conditions was 5.9 μSv. [19].

2.3. X-ray examination

During the X-ray diagnostics of sinuses the parameters of the irradiations were: X-ray voltage of 75 kV, 28 mA quantity of charge and 22 ms time of irradiation using an X-ray machine (Shimadzu CH-200 M unit, Japan). The conditions of exposure were normal for routine diagnostic procedures. The same technician performed all X-ray scans, thus avoiding inter-technician variability [17,20]. A protective lead apron on the neck was used to protect the thyroid gland.

2.4. Buccal micronucleus cyto assay

The buccal MN cyt assay was performed according to Thomas et al. [12] with minor modifications in which series of centrifugations and cytospin were performed to prepare the MN slides, as described by Milić et al. [21]. The slides were kept at –20 °C prior to staining and analysis. Scoring of slides was performed using transmitted light microscopy under far-red fluorescence at 1000× (Olympus BX-51, Japan). A minimum of 1000 cells were counted to determine the frequency of each cell type in the sample. Nuclear changes were classified according to HUMNxl criteria as: normal basal cells, normal differentiated cells, binucleated cells, cells with condensed chromatin, pyknotic cells, cells with karyorrhectic chromatin, and karyolytic cells. DNA damage biomarkers (cells with micronuclei and nuclear buds, including so-called "broken eggs" which represent nuclear buds with a diameter larger than 1/3 of the main nuclei) were scored in a minimum of 2000 differentiated cells [22].

2.5. Statistical analyses

Statistical evaluation was performed using STATISTICA 13.5.0.17 (TIBCO Software Inc., USA). Normality of distribution was tested with histograms, Kolmogorov-Smirnov, and Shapiro-Wilk tests. For the comparison of the group before and after the exam (N = 20), a Wilcoxon match paired test was used. The correlation of different parameters was done with Spearman's rank order correlations and the differences that reached p < 0.05 were considered significant.

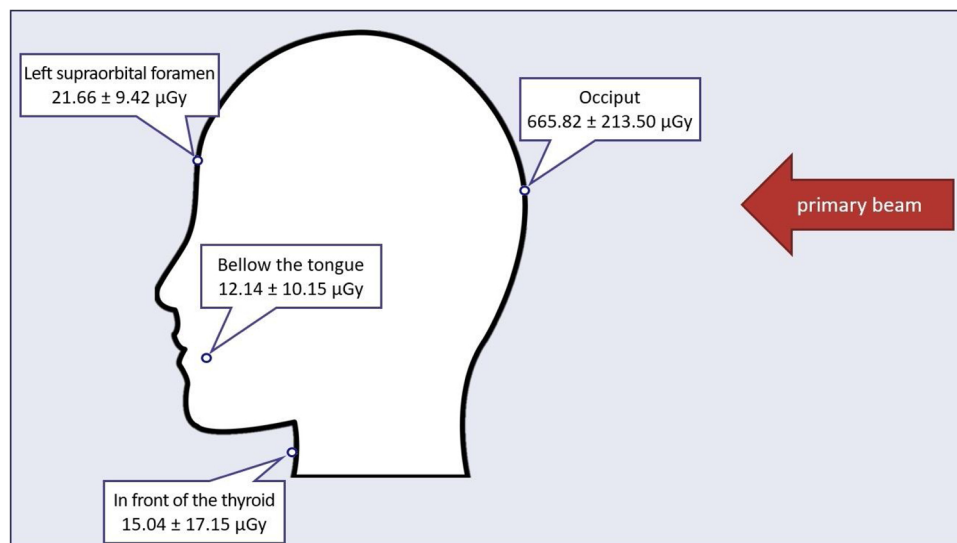


Fig. 1. The mean value and standard deviation of doses measured by RPL dosimeters on each positions: occiput, below the tongue, in front of thyroid, and on the left supraorbital foramen.

3. Results

3.1. Physical dosimetry

The average measured doses and their standard deviation for all 20 patients using RPL dosimetry systems at various positions on the head are presented in Fig. 1. The dose range at occiput in the primary beam was 371–1106 μGy, below the tongue 0–42 μGy, in front of thyroid 0–54 μGy, and on the left supraorbital foramen 10–39 μGy.

3.2. Buccal cells micronucleus cytome assay

The results of the buccal MN cyt assay are summarized in Fig. 2. In brief, there were no statistical differences for most of the parameters measured after the sinus X-ray exam. This applies to DNA damaging biomarkers (frequency of micronuclei, nuclear buds, and “broken eggs”), biomarkers tracking cytokinetic damage and proliferative potential (number of binucleated and basal cells), and biomarkers of cell death (number of karyorrhexis, pycnotic, and karyolytic cells). The only change observed was the significant ($p < 0.05$) increase in cells with condensed chromatin after the X-ray exam (51.7 ± 23.4 , compared to 41.9 ± 19.3 before the exam).

4. Discussion

To the best of our knowledge, no studies have yet investigated the effects of diagnostic paranasal sinus X-ray on buccal epithelia cells in pediatric population. As the children are a vulnerable population, and the buccal MN Cyt assay was developed to be less invasive in cell sampling, we decided to monitor changes upon diagnostic sinus X-ray. The only significant change detected in this study was the number of cells with condensed chromatin. Still, it should be noted that the results also revealed that inter-individual differences existed for each monitored child.

The rationale for performing an MN assay is reflected in its sensitivity and reliability to detect the effects of X-radiation of similar dose range in occupationally exposed adult and in pediatric populations [23,24], in addition to the cancer predictive potential, confirmed in prospective and cross-sectional studies [7,8,25]. Choosing peripheral blood lymphocytes to perform an MN assay is the method of choice when detecting chromosomal damage, however the development of the assay for buccal epithelial cells provides several advantages including non-invasive cell sampling and high correlation with the lymphocyte MN assay [11].

Several studies have used a similar approach when evaluating dental X-ray effects on pediatric populations. The number of volunteers recruited in those studies ranged between 20 and 60. Most did not observe changes in MN frequency [26–31], whereas Preethi et al. [32] found an increase in MN frequency in children ($N = 40$) after bitewing and digital dental panoramic radiography. An MN increase was also observed in a study by Waingade and Medikeri [33], but only after including all-age volunteers achieving $N = 60$. At the same time, most of the studies found an increase in the number of biomarkers related to cell death. These results support ours where the only significant change was observed for cells with condensed chromatin. The characteristic nuclear morphology is a reflection of chromatin aggregating in some regions of the nuclei, thus being lost in other regions, which is probably due to the initial stages of apoptosis [12]. Apoptosis can be triggered by either intrinsic or extrinsic stimuli and usually results in morphological changes including chromatin condensation, but also nucleus fragmentation and formation of apoptotic bodies. Moreover, apoptosis is a well regulated cell death process that is initiated upon cell damage, but also a part of normal development, unlike necrosis that is usually an acute response to the stress followed by inflammatory reactions [34–37].

Another strength of our study is the physical dosimetry per X-ray scan, which enabled us to track the dose distribution at different positions on the child's head. Observed doses were in accordance with our previous results using child phantom and pediatric patients [38,39]. The relatively high deviation of measured doses was caused by various factors. The most important reason, which is always present in X-ray diagnostics, is the variety of the patients' physical characteristics. In spite of our efforts to keep the possible parameters constant, (X-ray unit, irradiation conditions, technician), due to the variety of physical characteristics of the patients, the radiation absorption, the amount and the energy of the scattered radiation were different. The results of energy dependence on a phantom with GD-352M dosimeters containing a Sn filter can influence the dose measurements in X-ray diagnostics [19]. This research on child patients during X-ray diagnostics of the sinuses also revealed that routine X-ray diagnostics dosimetry measurements are a very useful tool for showing no changes or significant increases observed in radiation protection measures. A previous paper confirmed that the RPL dosimeters used in X-ray diagnostics give satisfactory agreement results compared to thermoluminescence (TLD) dosimeters [23].

We also performed a correlation for each buccal MN cyt assay parameter and detected dose, but found no significant correlations. The bystander effect such as gap junction cell-to-cell communication and production of soluble factors by irradiated cells, as well as adaptive

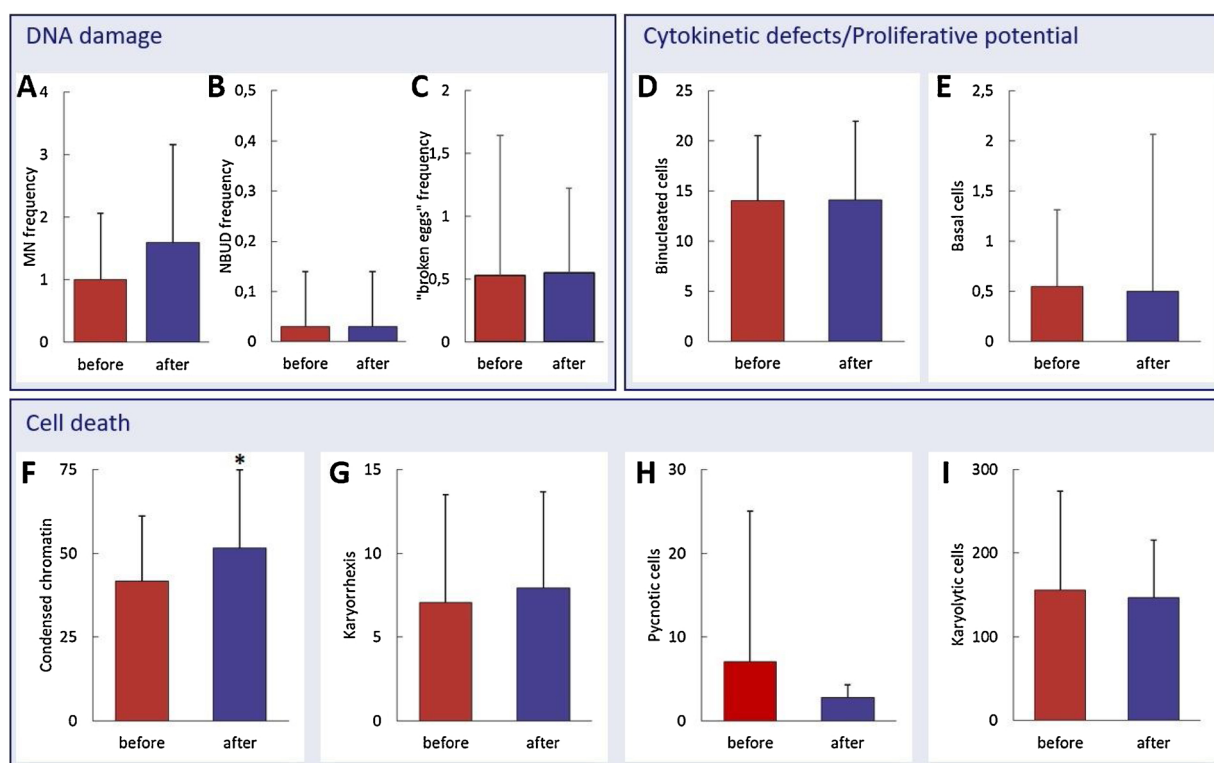


Fig. 2. The mean values \pm SD of buccal micronucleus cytome assay parameters for DNA damage: (A) frequency of micronuclei (MN) per 1000 cells, (B) frequency of nuclear buds (NBUD) per 1000 cells, (C) frequency of "broken eggs" per 1000 cells; for cytokinetic defects: (D) binucleated cells; for proliferative potential: (E) basal cells; and for cell death: (F) cells with condensed chromatin, (G) karyorrhexis, (H) pycnotic cells, and (I) karyolytic cells. *significant difference (at $p < 0.05$). DNA damage biomarkers were analysed on 2000 cells, while cytokinetic defects/proliferative potential and cell death biomarkers were analysed on 1000 cells.

response of the cells can at least partially explain why there was no linearity in observed effects [40]. Moreover, the dose-response curves may also not be linear in the low-dose area [41] and we examined only the small dose range of ~ 0.7 mGy in the primary beam of radiation (occiput) and even smaller ranges for other dosimetry locations.

Based on these results, it is safe to say that this study adds another brick to the complex puzzle of low-dose ionizing radiation effects. This is quite important for clinicians, particularly those working with pediatric patients, because our results might be useful in communicating health risks with the patient's parents. Improving the level of education for staff should lead to better outcomes, crucial for appropriate patient care. In other words, in keeping radiation doses as low as reasonably achievable (ALARA) and maintaining the benefits as high as reasonably achievable (AHARA) [42,43].

5. Conclusion

To conclude, according to the results in our experimental conditions, the diagnostic sinus X-ray did not induce DNA damaging effects in a pediatric population using buccal MN Cyt assay. The doses at the primary beam entry were the highest (up to 1.1 mGy), whereas below the tongue they ranged 0–42 μ Gy. The only difference detected in the volunteers approximately 14 days after the X-ray scan was the number of cells with condensed chromatin, reflecting cells in the early stages of apoptosis. The strength of the study was its simultaneous application of physical dosimetry and biological monitoring. The limit of the study was the group size, however it nevertheless brought valuable data on a specific, clinically relevant population. Further studies are needed to increase the statistical power of the results with an aim to secure patients' safety and optimal health care, as well as to estimate low-dose response calibration curves.

CRediT authorship contribution statement

Mirta Milić: Investigation, Methodology, Formal analysis, Resources, Writing - review & editing. **Marko Gerić:** Investigation, Methodology, Formal analysis, Resources, Writing - original draft. **Marijana Nodilo:** Investigation, Methodology, Formal analysis, Writing - review & editing. **Mária Ranogajec-Komor:** Investigation, Methodology, Formal analysis, Resources, Writing - review & editing. **Đurđica Milković:** Conceptualization, Investigation, Methodology, Formal analysis, Resources, Writing - review & editing. **Goran Gajski:** Conceptualization, Investigation, Methodology, Formal analysis, Resources, Writing - original draft.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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