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# DNA REPAIR PROCESSES IN HUMAN LYMPHOCYTES IRRADIATED WITH A 60-MeV PROTON RADIOTHERAPEUTIC BEAM

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***Abstract.*** *One of the main factors that differentiate people in terms of the effectiveness of therapeutic procedures or side effects is the variability in DNA repair capabilities. The aim of the study was to investigate the response of DNA damage repair systems in human lymphocytes irradiated with the therapeutic proton beam in the Bronowice Cyclotron Center of Institute of Nuclear Physics, Polish Academy of Sciences (IFJ PAN) in compare to X-rays. Lymphocytes from healthy donors were irradiated in the Spread-Out Bragg Peak of the proton beam or as reference X-rays. For both sources of radiation, the kinetics of the DNA damage repair capabilities were estimated using the comet assay method (0–120 min) and γ-H2AX test (0-24h). Preliminary results from the comet assay show a similar time and repair efficiency of induced DNA damage for both types of radiation. However, in a group involving X-rays, significant inter-individual differences were observed. With the γ-H2AX test, inter-individual differences in the repair capabilities were not noted. These findings indicate that induced DNA damage repair mechanisms after proton irradiation may be different when compared to X-rays.*

***Key words:*** *Protons, X-rays, DNA damage response/repair*

## 1. INTRODUCTION

Radiation therapy continues to be a mainstay in the treatment of a variety of cancers. Despite various therapeutic improvements, efficient radiation therapy of malignant cancers is limited by the adverse side effects occurring in the normal tissue when exposed to radiation [1], [2]. The use of proton beams is becoming a more attractive option for cancer therapy. It gives the precise absorbed dose-localization and an increased biological effectiveness on the tumor as well as sparing the surrounding normal tissues, in contrast to conventional treatment modalities delivered by photons or electrons [3], [4].

Exposure to ionizing radiation (IR) results in a variety of DNA lesions that include single strand breaks (SSBs), double strand breaks (DSBs), and a great variety of base damages [5]. DSBs are the most toxic form of DNA damage, because a single unrepaired DSB can lead to abnormal mitosis with losses of large fragments of DNA [6]. The repair of radio-induced DNA damage seems to be a key factor differentiating people in terms of the therapeutic efficacy and the toxicity to surrounding tissues, because it contributes to genetic stability.

Our previous research evaluated the response of Human Peripheral Blood Lymphocytes (HPBL) to therapeutic proton radiation of 60 MeV, by studying the mode of cell killing and the frequencies of cytogenetic damages and then comparing the results with X-rays [7], [8]. The results of these studies showed the differences between protons and photons for the studied biomarkers and also inter-individual variations in the cell's response between donors, which might be clinically relevant.

The aim of this study was to evaluate the response of lymphocytes to different radiation qualities (proton and X-rays) by determining the DNA damage repair. For this purpose, the formation and rejoining of DSBs was determined by the immunofluorescent detection of γ-H2AX foci at different times in the post-irradiation period (0–24h). The DNA damage repair capabilities were obtained by an alkaline version of the comet assay.

## 2. MATERIALS AND METHODS

### *2.1. Samples*

Heparinized whole blood samples with a volume of 4 ml were collected from healthy donors: 3 women and 4 men between 36 and 56 years of age without a history of exposure to ionizing radiation, other than that necessary for routine medical diagnosis. The human bioethical committee of the Regional Medical Board in Krakow approved the informed consent form used in this study (No. 124/KBL/OIL/2013). Lymphocytes were separated using the Histopaque separation medium. The collected cells were suspended ($1 \times 10^6$ / ml) in a freezing medium (10% DMSO, 40% RPMI,

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50% fetal calf serum). The cryopreserved lymphocytes where stored in liquid nitrogen. Before ex vivo studies, the lymphocytes were defrosted. Cell viability was determined using the Trypan blue exclusion technique. The viability after thawing has been usually better than 97%.

### *2.2. Irradiation*

The samples of human lymphocytes isolated from peripheral blood were irradiated with 60-MeV protons or X-rays (250 kV). A non-irradiated part of the sample served as the control (0.0Gy). Lymphocytes designated to repair studies were suspended in 200 μl of cool RPMI medium in vials. Immediately before and after irradiation, the samples were stored in a cool box. The proton beam facility beam delivery, monitoring system, X-ray irradiation, and dosimetry have been previously described in detail [9]. Lymphocytes were exposed to a proton dose of 1 Gy for γ-H2AX assay and 4 Gy for comet. An average measured dose rate was 0.15 Gy/s. The samples were irradiated in SOBP with a range of 29 mm and a modulation of 30 mm. During irradiation, the plastic vials with the lymphocytes were placed inside a specially designed poly (methyl methacrylate) (PMMA) phantom in the mid of the SOBP and in the center of the flat beam field with a diameter of 40 mm. At the vial with sample position i.e. at the depths 14.5 mm in the SOBP the calculated Continuous Slowing Down Approximation (CSDA) dose averaged of the linear energy transfer (LETd) was 2.8 keV / μm. For X-ray irradiation, lymphocytes were exposed to the same dose as during proton irradiation, but at a rate of 0.063 Gy/s using a Philips X-ray machine (model MCN 323) at 250 kV, 10 mA operating at the IFJ PAN. After the exposure, FBS was added (40 μl per vial) to lymphocytes suspended in the RPMI medium and the cells were transferred into an incubation chamber (37 °C, 5 % $CO_2$) for various periods of time (0 – 120 min for the comet assay and 0 – 24h for the γ-H2AX test).

### *2.3. γ-H2AX test*

The DNA strand breaks were evaluated using a γ-H2AX test. The γ-H2AX foci were visualized after conjugation with anti-γ-H2AX antibodies. Shortly after irradiation, the cells were incubated for 0, 0.5, 2 and 24 hours, washed with PBS and fixed with 100% cool methanol. They were then permeabilized (0.5 % Triton X-100 solution in PBS) for 5 min and incubated in a blocking buffer (5 % bovine albumin serum in PBS) for 30 min at room temperature. After the preparatory stage, the cells were incubated with the anti-mouse anti-phospho-Histone H2AX (Ser139) antibody (Clone JBW301, Maus, Upstate, New York, USA) overnight, and thereafter with goat anti-mouse secondary antibodies conjugated with Alexa Fluor 488 (Vector Laboratories INC, Burlingame, CA, USA) for 1 hour at room temperature. Radiation-induced γ-H2AX foci were counted directly in 100 cells which nuclei were counterstained with DAPI (2 μg / ml) cells using a Zeiss epifluorescence microscope. Foci values were presented as mean foci/cell values and the standard deviations were calculated from the experiments with Poisson distribution mathematical assumption [10], [11]. The number of radiation-induced damage foci per cell was then obtained by subtracting the background focus rate for each sample. We tested the differences between protons and X-rays using other statistical methods i.e. ANOVA (Excel and MedCalc statistical software) by comparing CV values.

### *2.4. Comet assay*

The comet assay estimated DNA damage level on the base the electrophoretic migration of relaxed or fragmented DNA. The details of this procedure were reported elsewhere [12], [13]. Lymphocytes were lysed for 1h in the agarose by detergent (1 % Triton X-100) in alkaline pH > 13. Alkaline electrophoresis (30 V, and 300 mA) was then carried out for 30 min at 4°C. Cells were stained with ethidium-bromide (17 mg / ml). Cellular DNA was visualized using an epifluorescence (excitation filter 515–560 nm, barrier filter from 590 nm) microscope Olympus BX-50 connected with a CCD camera. The semi-automated image analysis system Komet 3.0 (Kinetic Imaging Company, Liverpool, UK) was used to evaluate the T-DNA parameter – tail DNA (DNA percentage in the comet tail) as a sensitive indicator of DNA repair. Two independent experimental replicates were performed for each aliquot: from 200 to 500 cells were analyzed for each data point (2 slides per each dose, 100–250 cells from each slide) in accordance with data published by Panek [14]. The kinetics of DNA damage repair curves performed in irradiated lymphocytes were fitted with the Slide Writer 3.0 software (Advanced Graphics Software). Statistical analyses were performed using a one-way analysis of variance (ANOVA), and significance was assumed if $p < 0.05$. We used Excel and MedCalc (MedCalc Software, Belgium) as a statistical software.

## 3. Results

DNA DSBs were visualized by immunofluorescent staining for γ-H2AX foci that were analyzed at various time points (30 min, 2, and 24 h) after irradiation with a dose of 1 Gy. The results of the γ-H2AX foci formation assay are shown in Fig. 1.

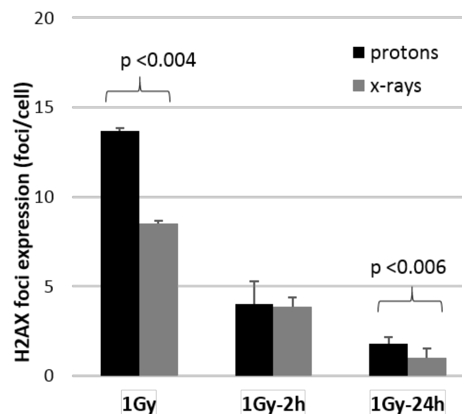


Figure 1. Formation and dissolution of γ-H2AX foci induced by 1 Gy of protons or X - rays. Each point represents the data from 7 donors per treatment group. Error bars indicate standard errors.

The mean value of the number of phosphorylated H2AX foci per cell before radiation (background) for all studied donors was 0.55 ± 0.12. The number of foci peaked after 30 min of the post-irradiation period (13.70 ± 1.30 after protons and 8.53 ± 0.52 after X-rays) and then decreased gradually over time up to the 24-hour mark. Thirty minutes after exposure, the lymphocytes from seven donors exhibited a statistically significant higher γ-H2AX foci expression ($p < 0.004$) response to 60-MeV protons in comparison to 250 kV X-rays. Also, after 24h of post-irradiation incubation, a similar effect was observed (1.79 ± 0.21 after protons and 1.03 ± 0.04 after X-rays; $p < 0.006$). These results confirmed Zlobinskaya's research carried out on HeLa cells, which showed a higher number of γ-H2AX foci after 1 Gy of 20-MeV protons in comparison to 200-kV γ-rays [15].

The radiation-induced % of T-DNA values as a function of repair time in the range of 0 to 120 min are shown in Figure 2. For each donor, the percent of T-DNA values measured in the non-irradiated cell population was subtracted from each level of DNA damage observed after irradiation. For both types of radiation, the obtained results showed a significant decrease in the DNA damage level in the first 30 min (for protons $p < 0.001$, $F = 2.39$ and for X-rays $p < 0.001$, $F = 2.38$). After 2h of post-irradiation incubation, a similar level of DNA damage was observed.

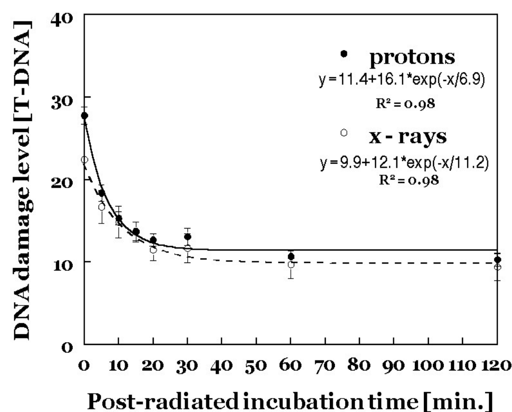


Figure 2. Residual DNA damage after 4Gy of protons or X-rays detected in various time points of post-irradiation incubation. Each point represents the data from 7 donors per treatment group. Error bars indicate standard errors.

To illustrate the repair processes of the DNA damage induced by radiation, the efficiency of the radiation-induced DNA damage repair parameter (RE) was calculated from the amounts of the DNA damage/foci detected in cells right after the irradiation and right after the post-irradiation incubation:

$$RE_{T-DNA} = \frac{T-DNA_{4Gy} - T-DNA_{120\,min}}{T-DNA_{4Gy}} * 100\% \quad (1)$$

$$RE_{\gamma-H2AX} = \frac{\gamma-H2AX_{1Gy} - \gamma-H2AX_{1Gy-24h}}{\gamma-H2AX_{1Gy}} * 100\% \quad (2)$$

where: $T\text{-}DNA_{4Gy}$ or $\gamma\text{-}H2AX_{1Gy\text{-}0.5h}$ represents the immediate response to radiation, $T\text{-}DNA_{120min}$ stands for the DNA damage or $\gamma\text{-}H2AX_{1Gy\text{-}24h}$ foci measured after the repair incubation

The experimental data were averaged for all studied individuals.

Table 1. The repair efficiency of the radiation-induced DNA damage detected using the γ-H2AX foci test and the comet assay.

| Radiation | | RE $_{\gamma\text{-H2AX}}$ | RE $_{T\text{-DNA}}$ |
|---|---|---|---|
| 60-MeV protons | Mean | 86.6 | 84.4 |
| | Range | [78.5–90.6] | [72.4–90.9] |
| | CV | 1.8 | 8.8 |
| 250-kV X-rays | Mean | 87.6 | 82.7 |
| | Range | [86.8–92.1] | [45.1–100.0] |
| | CV | 1.0 | **24.4** |

RE $_{\gamma\text{-H2AX}}$ – the efficiency of the radiation-induced γ-H2AX foci repair calculated from equation 1; RE $_{T\text{-DNA}}$ – the efficiency of the radiation-induced DNA damage repair detected by the comet assay and calculated from equation 2; CV – coefficient of variation

The results of the repair capabilities are shown in Table 1. It can be seen that the mean values of the repair efficiency estimated from the DNA damage detected that both applied techniques are similar. There is no significant difference between protons and X-rays. Only for comet assay method, in lymphocytes exposed to X-rays did the coefficient of variation show a greater variation (CV = 24.4) in DNA repair between donors (Figure.3).

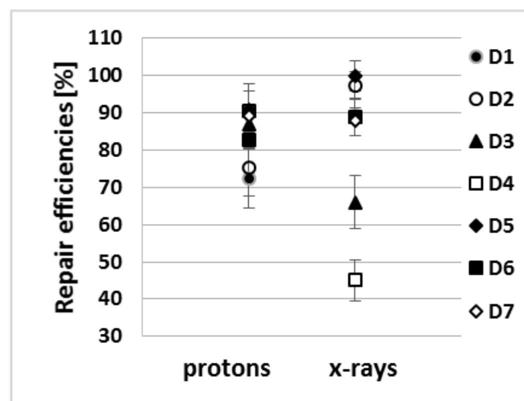


Figure 3. Frequency distribution of the efficiency of the radiation-induced DNA damage repair parameter (RE) for the 7 donors detected by use of comet assay.

Differences in the repair of DNA damage may result from various spectrum DNA damage detected by both used methods. Γ-H2AX test detects only DSBs while the alkaline version of the comet shows a wider spectrum of damages: DSBs, SSB as well as alkali-labile (AP) sites. These differences may also result from the kinetics of repair, because the comet assay was analyzed only after 2 hours, (that's mainly concerns quickly repairable DNA damages), while H2AX concerns the DSB repair monitored by 24 h. Such variations of radiation-induced DNA damage repair capabilities in human lymphocytes have been seen in several studies both in groups of healthy donors and cancer patients [16], [17]. Different cell reaction of the

same individuals on proton and X-ray radiation might be a result of many factors, including the various types and complexity of DNA damages which affected survival, proliferation, cell-cycle alterations, gene-expression changes, and the secretion of signaling factors [18], [19]. The results of our investigation may suggest that the differences between patients can largely be related to the activation of repair systems responsible for the repair of damage other than double-strand breaks.

## 5. Conclusion

Although a limited number of donors was studied, the obtained results show potential mechanisms determining various cellular response on radiation. In this context, the individual radiation treatment based on the DNA damage repair capability could be important for patients qualifying for therapy using different radiation types. Moreover, it seems that the potential advantage of protons might be associated with less intra-individual variability in DNA repair between different patients.

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