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Keywords : DNA damage, oxidative stress, carbon ions, skin, 8-oxodG

Color not necessary in print.

Manuscript has been 'spell checked' and 'grammar checked'.

The authors report no conflict of interest.

ABSTRACT

This work aims to evaluate genotoxicity of C-ion *vs.* X-ray irradiation in normal human skin fibroblasts. Clonogenic cell survival was first evaluated using a linear-quadratic model. Then, early and late genotoxicity was quantified by alkaline comet assay, micronucleus test and 8-oxodG extracellular measurement. Survival strongly decreased after C-ions compared to X-rays with a 4.8-fold decrease at D_0 – irradiation dose corresponding to 37% of survival. The level of immediate DNA damage was approximately the same after C-ions or X-rays. However, half-time of DNA repair was 1.3-fold decreased after C-ions compared to X-rays leading to a 2.2-fold increase in remaining damage. In the same way, micronucleus formation was 1.7-fold increased 24 hr after irradiation and remained 1.8-fold increased 2 weeks after C-ions *vs.* X-rays when secondary oxidative stress wave occurred. This was related to a 2.6-fold increase in binucleated cells percentage in carbon- *vs.* X-ray-irradiated fibroblasts. Excretion of 8-oxodG was also 1.5–fold increased after C-ions *vs.* X-rays. 8-oxodG excretion could therefore participate to the appearance of late waves of oxidative stress. These results showed a stronger genotoxicity of C-ions in skin fibroblasts. More investigations are needed to clarify the discrepancy between the two types of radiations.

1. INTRODUCTION

The field of hadrontherapy is of growing interest for cancer treatment. Protons and carbon ions are the most used hadrons. While proton effects on tumours have been investigated since decades – showing a more precise dose delivery and a biological effectiveness evaluated at 1.15 compared to conventional radiation therapy (Paganetti et al., 2002) – the use of C-ions is even more recent and still needs to be investigated.

It is considered that C-ion biological effectiveness on tumours is from to 2 to 3 compared to proton therapy (Suit et al., 2010). Moreover, literature on C-ion effects on normal tissues is relatively poor. Skin being the first organ exposed to irradiation, some studies reported skin

toxicity after C-ion therapy (Imai et al., 2004; Mizoe et al., 2004; Nomiya et al., 2008; Rieber et al., 2015; Schulz-Ertner et al., 2002; Tsujii et al., 2007; Zhang et al., 2012). In particular, Yanagi et al. (2010) reported severe acute and late skin complications on patients treated for unresectable bone and soft tissue sarcoma by a C-ion dose escalation protocol. We have recently reported that skin fibroblasts showed a stronger increase in oxidative stress after C-ion vs X-ray irradiation, even at late times - two weeks - after *in vitro* irradiation (Laurent et al., 2013). As DNA is considered the main target of radiation by direct or indirect effects but also the target of oxidative stress occurring by waves after irradiation, the early and late increased oxidative stress that was observed in skin fibroblasts (Laurent et al., 2013) could lead to a stronger genotoxicity of C-ions compared to X-rays. Some previous studies showed an increase in DNA damage measured by alkaline comet assay or micronuclei formation after C-ions compared to X-rays (Autsavapromporn et al., 2013; Fujisawa et al., 2015; Ghorai et al., 2014; Pathak et al., 2007). However, these works were not performed on primary skin cells and at late times after irradiation. Our previous work also demonstrated that nucleus was not the only target of C-ions, especially 24 hr and 2 weeks after irradiation when a secondary wave of oxidative damage occurred (Laurent et al., 2013). Extracellular 8-oxo-7,8-dihydro-2'- deoxyguanosine (8-oxodG) – which is the most abundant oxidized nucleoside (Ravanat et al., 2012) – is considered as mainly coming from the cytoplasmic nucleotide pool which is an important target of oxidative stress (Haghdoost et al., 2006; Sangsuwan and Haghdoost, 2008). Oxidized extracellular DNA could be therefore a biomarker of major interest. Indeed, the excretion of damaged DNA could be at the origin of bystander effects and secondary waves of ROS (for review, see Glebova et al., 2015). In this way, the measurement of excreted 8-oxodG could give information on oxidative waves occurring after C-ion irradiation. The biological role of extracellular DNA remains unclear. Indeed, DNA in cell culture supernatants could come from cell death after irradiation (Jahr et al., 2001). This released DNA could participate to bystander effects and/or to secondary waves of ROS. During radiation therapy, the release of oxidized DNA could be responsible in patients for adverse effects occurring at distance from irradiated area (abscopal effects). Studies are necessary to understand and eventually modulate the level of oxidized extracellular DNA using response-adapted radiotherapy.

In the present work, we investigated early and late DNA damage produced by C-ions in human skin fibroblasts from young adult healthy individuals. Fibroblasts were irradiated at confluence (G_0) to mimic skin physiology, either with X-rays (15 MV) or with C-ions (72 MeV/n; LET = 33.6 keV/µm) (Pautard et al., 2008) at isosurvival irradiation doses. This C-ion energy delivered at the GANIL facility (Caen, France) matches the delivered dose in the plateau-phase before

the Spread-Out Bragg Peak (SOBP). Genotoxicity was measured immediately and 2 weeks after irradiation as these endpoints were those when waves of oxidative stress were observed (Laurent et al., 2013).

2. MATERIALS AND METHODS

2.1.Reagents

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) buffer, Dulbecco's phosphate-buffered saline (PBS), trypsin/EDTA, L-glutamine, penicillin and streptomycin were purchased from Gibco BRL Life Technologies (Gaithersburg, MD, USA). 8-hydroxy-2′-deoxyguanosine (8-oxodG) was purchased from Calbiochem® (LaJolla, CA, USA). Methanol HiPerSolv Chromanorm® HPLC grade was purchased from VWR International S.A.S (Fontenay-sous-Bois, France). All the others chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2.Cell cultures

Primary cultures of normal human dermal fibroblasts (Lonza, Verviers, Belgium) were grown in DMEM supplemented with 20% FBS, 100 µg/mL L-glutamine, 10 mM HEPES and antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin), at 37°C in a humidified atmosphere containing 5% CO_2 and were used in passages 4-7.

2.3.X-ray and C-ion irradiation

Confluent cells were irradiated at high isosurvival doses: 10 Gy for X-rays and 6 Gy for C-ions. Irradiations were performed at room temperature either with X-rays using a Saturne 15 generator (GE-CGR MeV, Riverside, CA, USA; 15 MV, 1 Gy/min) or with C-ions on the D1 line of the GANIL accelerator (Caen, France; 75 MeV/n, 1 Gy/min). Cells were kept up to two weeks after irradiation and medium was replaced twice a week.

2.4.Clonogenic survival

Clonogenic survey of the cells was assessed as described previously (Laurent et al., 2013). Briefly, a defined number of cells, ranging from 1000 to 5000 fibroblasts for 7 irradiation doses between 0 and 6 Gy, was plated in 25 cm^2 tissue culture flasks 18 hr after irradiation. Cells were then grown for 10 to 14 days. Colonies were stained and those containing 50 cells or more were scored and the surviving fraction was calculated.

2.5.Alkaline single-cell gel electrophoresis

The alkaline single-cell gel electrophoresis assay (comet assay) was used to measure early single- and double-strand breaks together with alkali-labile sites in the nucleus DNA of irradiated confluent fibroblasts exposed to 6 Gy of C-ions or 10 Gy of X-rays. The assay used was described by Laurent et al. (2005). The mean Olive Tail Moment (OTM) immediately, 1 hr or 3 hr after irradiation was calculated using the computer image analysis software CASP. For each experimental condition, the mean OTM value of 50 cells per slide was determined.

2.6.Micronucleus assay

Micronucleus assay was used to measure chromosomal loss and cell cycling capacity. For this purpose, confluent primary skin fibroblasts are reseeded 24 hr and 2 weeks after exposure to C-ions at 6 Gy *vs.* X-rays at 10 Gy. The assay used was described by Laurent et al. (2005). Briefly, cells were reseeded at different time points. Twenty-four hours after they were seeded, cytochalasin B was added to the culture medium in order to block cytokinesis and maintained for 24 hr later. Cells were then treated with KCl for hypotonic chock. Cells were thus fixed. The number of micronuclei and binucleated cells was determined among 500 cells using a fluorescence microscope.

2.7.Quantification of extracellular 8-oxodG in cell culture supernatant

Extracellular 8-oxodG was quantified in confluent skin fibroblasts 24 hr and 2 weeks after exposure to C-ions at 6 Gy *vs.* X-rays at 10 Gy. Prior to analysis, the cell culture supernatant (0.5 mL) was purified by solid phase extraction (SPE) ; samples were adjusted for the standard addition method in order to correct matrix effects contributed by the culture medium constituents (Lagadu et al., 2010). An optimised method for the quantification of 8-oxodG in white blood cell DNA hydrolysate has been applied and slightly modified from Breton et al. (2005). Briefly, the SPE eluate was analysed by HPLC with an electrochemical detector (Coulochem III, ESA Inc., Chelmsford, MA, USA) using an Uptispher ODB C18 HPLC column (150 × 2.1 mm, 3 μm; Interchim, San Pedro, CA, USA) equipped with a C18 guard column. The eluant was 10 mM potassium dihydrogen phosphate, pH 4.6, containing 11.2% methanol, at a flow rate of 0.2 $mL.min^{-1}$. The potentials applied to the analytical cell (ESA 5011) were + 50 mV and + 350 mV for E1 and E2, respectively. Acquisition and quantitative analyses of chromatograms were carried out using Azur v 4.6 software (Datalys, St Martin d'Heres, France). HPLC-ECD signals were recorded in the culture supernatants spiked with the

external standard and values for 8-oxodG peak areas plotted against 8-oxodG concentration (in nM). The linear plot thus obtained was extrapolated to determine the value of the *x*-intercept, which represents the absolute concentration of 8-oxodG in the test samples.

2.8.Statistical analysis

Data are depicted as mean ± SEM. ** for $p<0.001$ or * for $p<0.05$ for irradiated cells compared with control cells and †† for $p<0.001$ or † for $p<0.05$ for C-ion compared with X-ray irradiated cells (one-way ANOVA with Tukey test). Each biological experiment was performed three times independently, and technical measurements were performed in triplicates.

3. RESULTS

3.1. Survival

Skin fibroblast survival was measured by clonogenicity assay (Figure 1). Surviving fraction (S) was defined as the fraction of surviving cells after an irradiation dose (D). Plating efficiency of control cells was measured and taken into account to calculate S as:

$S = S(D)/S(0)$. S were fitted on the linear-quadratic model with $\ln(S) = -(\alpha D + \beta D^2)$. Alpha values were 25-fold increased after C-ion compared to X-ray irradiation corresponding to a strong decrease in survival. Furthermore, β component was close to 0 after C-ion irradiation with a 3-fold decrease after C-ion compared to X-ray irradiation. For the following experiments, isosurvival irradiation doses were chosen: 10 Gy X-rays and 6 Gy C-ion. Theses doses correspond to an almost zero survival in dividing cells.

Figure 1. Primary skin fibroblast survival after X-ray or C-ion irradiation. Top panel: clonogenic survival curves according to the linear-quadratic model; Bottom panel: linear-quadratic curve parameters, α (Gy^{-1}), β (Gy^{-2}), r^2. Each experiment was performed three independent times in triplicate.

3.2. *DNA strand-breaks and alkali-labile sites*

Alkaline single-cell gel electrophoresis assay gives an overview of DNA single- and double-strand breaks as well as alkali-labile sites (Figure 2). Olive Tail Moment (OTM) was considered as a reliable parameter as it takes into account the gravity center of the comet tail. OTM are calculated from the equation:

OTM = (CG-CGH)x(DNA/100) where CG is the center of gravity of the tail or body weighted by gray values, CGH is the center of gravity of the head weighted by gray values and DNA is tail or body DNA.

OTM were increased immediately after X-ray or C-ion irradiation in the same manner. However, the slope of repair was stronger for X-rays than for C-ions. Indeed, 1 hr after irradiation, OTM value remains 2.2-fold higher after C-ions than after X-rays. In this way, mean half-time of DNA repair was 1.3-fold higher for C-ions. Three hours after irradiation, OTM values remain 1.6-fold higher after both types of irradiation compared to their respective controls.

Figure 2. DNA damage measured by alkaline comet assay in primary skin fibroblasts irradiated by X-rays or C-ions. Top panel: repair curves; Bottom panel: slopes and half-times of DNA repair. Data are depicted as mean ± SEM. ** for $p<0.001$ or * for $p<0.05$ for irradiated cells compared with control cells and †† for $p<0.001$ or † for $p<0.05$ for C-ion compared with X-ray irradiated cells (one-way ANOVA with Tukey test). Each experiment was performed three independent times in triplicate.

3.3. Micronucleus frequency

Skin fibroblast micronuclei reflecting chromosomal damage were counted (Figure 3 A). Twenty-four hours after X-ray or C-ion irradiation, micronucleus frequency was 4.2-fold and 9.2-fold increased, respectively. Control micronucleus frequency was approximately the same for both types of radiation. Micronucleus formation increased to a 18.7-fold and a 40.1-fold two weeks after irradiation concerning X-rays and C-ions, respectively. In this way, C-ion micronucleus frequency was 1.7-fold or 1.8-fold higher than X-ray micronucleus frequency at 24 hr or two weeks after irradiation, respectively.

3.4. Percentage of binucleated cells

Percentage of binucleated cells represents skin fibroblasts cell ability to divide (Figure 3 B). After irradiation, percentage of binucleated cells was 2.5-fold or 1.7-fold decreased after X-rays or C-ions, respectively. Interestingly, percentage of binucleated cells continued to decrease after X-ray exposure, reaching 3.6-fold less than controls whereas it was 2.6-fold higher in carbon irradiated cells.

Figure 3. Micronucleus frequency (panel A) and percentage of binucleated primary skin fibroblasts (panel B) after X-ray or C-ion irradiation. Data are depicted as mean ± SEM. ** for $p<0.001$ or * for $p<0.05$ for irradiated cells compared with control cells and †† for $p<0.001$ or † for $p<0.05$ for C-ion compared with X-ray irradiated cells (one-way ANOVA with Tukey test). Each experiment was performed three independent times in triplicate.

3.5. 8-oxodG excretion

8-oxodG concentrations in culture medium supernatants were measured by HPLC-ECD 24 hr and two weeks after for both types of irradiation (Figure 4). Excretion of the oxidized nucleoside 8-oxodG in supernatants was increased in the same order of magnitude for both studied times. A 2.6-fold or 3.8-fold increase was observed for X-ray or C-ion irradiation, respectively. Levels of 8-oxodG were 1.5-fold higher after C-ion versus X-ray irradiation.

Figure 4. 8-oxodG concentration in supernatants of primary skin fibroblast cultures after X-ray or C-ion irradiation. Data are depicted as mean ± SEM. ** for $p<0.001$ or * for $p<0.05$ for irradiated cells compared with control cells and †† for $p<0.001$ or † for $p<0.05$ for C-ion compared with X-ray irradiated cells (one-way ANOVA with Tukey test). Each experiment was performed three independent times in triplicate.

4. DISCUSSION

Carbon therapy being in development, some studies pointed out the toxicity of these particles in terms of healthy tissue toxicity as well as the induction of secondary tumors (Ishikawa et al., 2015; Jensen et al., 2015; Mizoguchi et al., 2014; Morimoto et al.,2014; Wakatsuki et al., 2014;Yanagi et al., 2010). To understand the origins of these effects of C-ions, we dealt with oxidative damage which may explain the appearance of late cutaneous damage. In this study, we investigated genotoxicity of C-ions in comparison with X-rays in primary cultures of normal skin fibroblasts.

Skin fibroblasts were exposed to 33.6 keV/µm C-ion beams (Pautard et al., 2008). This LET corresponding to plateau-phase before SOBP in C-ion RT was adapted to our experiments on dermal fibroblasts as skin is the first organ exposed at the entrance of the beam.

For genotoxicity measurements, skin fibroblasts were kept at confluence (G_0), as it is the case in skin. High irradiation doses – 6 Gy for C-ions compared to 10 Gy for X-rays – corresponding to an almost zero survival rate in dividing cells were chosen to emphasize phenomenon that

could occur *in vivo*. Moreover, dose escalation protocols are often used for C-ion therapy since hypofractionation is one of the main advantages of C-ion therapy.

Skin fibroblast survival was strongly decreased after C-ion compared to X-ray irradiation with a RBE value of 4.8 at D_0 – the dose corresponding to a surviving fraction of 37% – and 3.3 at $D_{10\%}$, RBE corresponding to the ratio: X-ray value/C-ion value. This $RBE_{10\%}$ is higher than the mean of RBE – from 2 to 3 – observed in tumor cells (Suit et al., 2010) which is not in favour of a lower toxicity of C-ion therapy rather than conventional radiotherapy. The alpha component of linear quadratic survival curves which is strongly increased after C-ions compared to X-rays represents the initial slope and is mostly due to double-strand breaks. Alpha value is very high after C-ion irradiation depicting an almost linear curve at low doses. In this way, radiosensitivity of skin fibroblasts is strongly increased after C-ion exposure compared to X-rays. Furthermore, beta component - considered as representing double-strand breaks resulting from coincident single-strand breaks - was lower and almost zero for carbon irradiated cells. This is in agreement with high LET irradiations as they produce clusters of double-strand breaks difficult to repair.

In this way, immediate global DNA damage measured by alkaline comet assay were not higher after C-ion *vs*. X-ray irradiation. This could be due to the production of smaller DNA fragments (multiple damaged sites) which could reduce the quantity of DNA in the comet tail detected by comet assay. As expected, DNA damage produced after C-ion irradiation were more complex to repair. Indeed, half-time of DNA repair was 2.2-fold higher after carbon irradiation compared to X-rays. Wang et al. (2008) have shown that DNA repair by NHEJ (Non-Homologous End Joining) pathway was inhibited after C-ion irradiation as high-LET radiations induced smaller fragments inhibiting the efficient binding of Ku to DSB fragments. In the same manner, micronucleus frequency described as a result of impaired repair of DNA double-strand breaks was 1.7-fold increased 24 hours after carbon irradiation compared to X-rays. Interestingly, the same increase (1.8-fold) persisted at long term – 2 weeks after irradiation – where a late wave of oxidative damage was previously observed (Laurent et al., 2013). Increases in micronucleus frequency after X-rays could be related to decreases in cell dividing ability as measured by binucleated cell percentage as X-irradiated fibroblasts tend to undergo premature senescence (for review, see Suzuki and Boothman, 2008). In this way, remaining cells able to divide would present a lot of DNA damage and would undergo mitotic death. However, it is not the case for C-ions as no significant decreases in binucleated cell percentage compared to their controls were observed, which is probably due to a decrease in cell cycle arrests. Early and late stronger increases in micronucleus frequency after C-ions *vs*. X-rays should come from a smaller

proportion of cells undergoing premature senescence so that a bigger proportion of strongly damaged cells are able to divide and produce micronuclei. Investigations are needed to confirm this hypothesis. Surprisingly, comet OTM values of C-ion controls at 1 and 3 hr are higher than X-ray controls, this could be due to C-ion irradiation conditions as experiments with control cells were performed in the same manner than irradiated cells.

The appearance of a stronger late wave of oxidative damage 2 weeks after C-ion irradiation (Laurent et al., 2013) and confirmed by micronucleus assay could come from extracellular oxidized DNA. Indeed, extracellular 8-oxodG has already been a candidate for a prognostic factor in oncology (Roszkowski et al, 2005) and used as a marker of effects after radiotherapy *in vitro* (Glebova et al., 2015; Haghdoost et al., 2005) and *in vivo* (Skiöld et al., 2013; Haghdoost et al., 2005). Oxidized extracellular DNA could also be a signalling factor in bystander effects (Ravanat et al., 2012). 8-oxodG is the main oxidatively generated DNA lesion and is formed either by direct oxidation or can be incorporated in DNA from oxidized nucleotide pool by DNA polymerase (Kasai, 2016; Speina et al. , 2005). Its extracellular formation gives an overall view of DNA oxidation in both cytoplasmic nucleotide pool and nuclear DNA (Glebova et al., 2015; Haghdoost et al., 2005). Its extracellular presence can be due to DNA repair, cell death, mitochondrial turnover, cellular uptake or salvage of DNA damage products. After irradiation, 8-oxodG secretion would not come mainly from apoptotic or necrotic death or from DNA repair but from the nucleotide pool (Glebova et al., 2015; Haghdoost et al., 2005). Indeed, 8-oxodG excretion was increased after both types of irradiation and for both studied times, when no cell death was observed. Skin fibroblasts were kept at confluence so that released oxidized DNA could not come from dying cells. Extracellular 8-oxodG was 1.5-fold risen 24 hr and 2 weeks after C-ion beam exposure compared to X-rays. 8-oxodG secretion comes mainly from nucleotide pool - mostly located in the cytoplasm - and C-ions seemed to be more efficient to induce oxidative stress in fibroblasts. Both of these arguments reinforce our assumption that this emphasized increase in 8-oxodG after C-ion irradiation could be explained by stronger waves of not detoxified ROS occurring in the cytoplasm. We can hypothesize that early production of extracellular oxidized DNA could produce secondary waves of ROS maintaining high extracellular damaged DNA. This could occur in bystander cells but also in already irradiated cells. Further works are needed to understand the precise role of this released DNA in the occurrence of waves of oxidative stress after irradiation and, more generally, in abscopal effects observed in patients after radiotherapy. Our results motivate further investigation regarding the mechanisms underlying the oxidative damage after radiotherapy and the possible use of extracellular 8-oxodG as predictive marker of acute healthy tissue side effects. In this

way, modulation of extracellular DNA using tailored radiotherapy – taking into account individual variations towards the effects of radiation-induced oxidative damage – could be an interesting research pathway for clinical pratice.

ACKNOWLEDGMENTS

We would like to thank the Radiation Therapy and Physics Departments of the CLCC Baclesse, CIMAP staff for irradiations at GANIL facility, Violaine Mazur for helpful reading of the manuscript and Pr Khaled Meflah, head of CLCC Baclesse. This work was supported by grants from Electricité de France and the Ligue contre le Cancer.

Abbreviations:

8-oxodG: 8-oxo-7,8-dihydro-2′-deoxyguanosine

C-ions: carbon ions

HPLC-ECD: High-performance liquid chromatography coupled with electrochemical detection

LET: Linear Energy Transfer

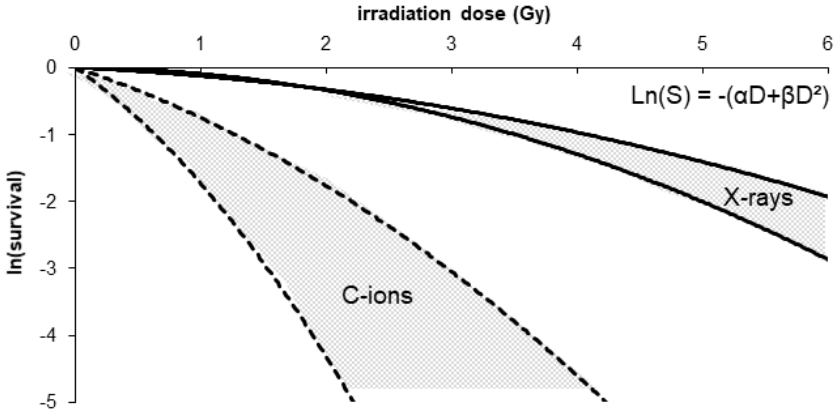
OTM: Olive Tail Moment

RBE: Relative Biologial Efficiency

ROS: Reactive Oxygen Species

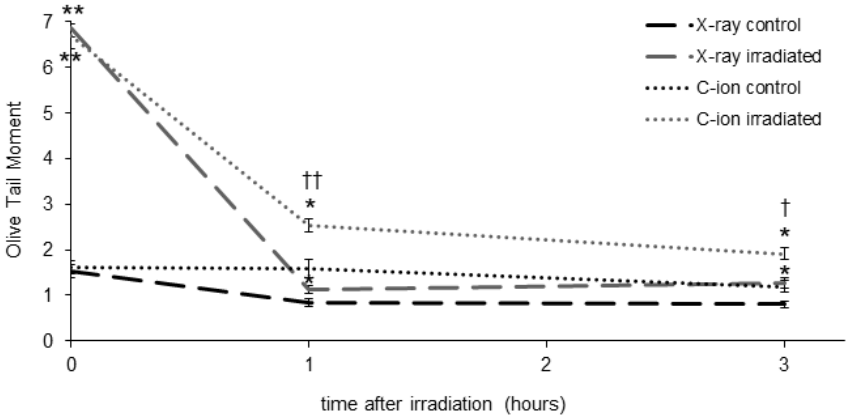
SOBP: Spread-Out Bragg Peak

SPE: Solid-Phase Extraction



	α	β	r²
X-rays	0.0485 ± 0.0049	0.0585 ± 0.0141	0.98
C-ions	1.2526 ± 0.2182	0.0188 ± 0.0348	0.99

Figure 1



	slope	half-time DNA repair
X-rays	-5.72	35.97 min
C-ions	-4.17	48.17 min

Figure 2

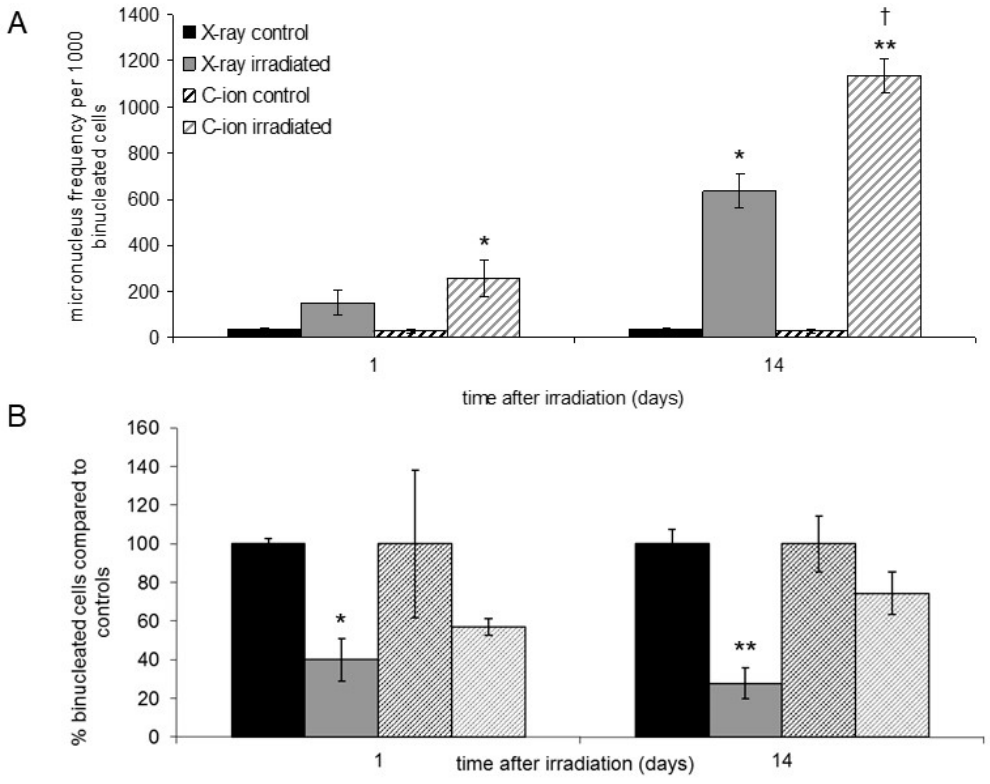

A
micronucleus frequency per 1000 binucleated cells
X-ray control
X-ray irradiated
C-ion control
C-ion irradiated
1400
1200
1000
800
600
400
200
0
*
*
**
†
1
14
time after irradiation (days)
B
% binucleated cells compared to controls
160
140
120
100
80
60
40
20
0
*
**
1
14
time after irradiation (days)

Figure 3

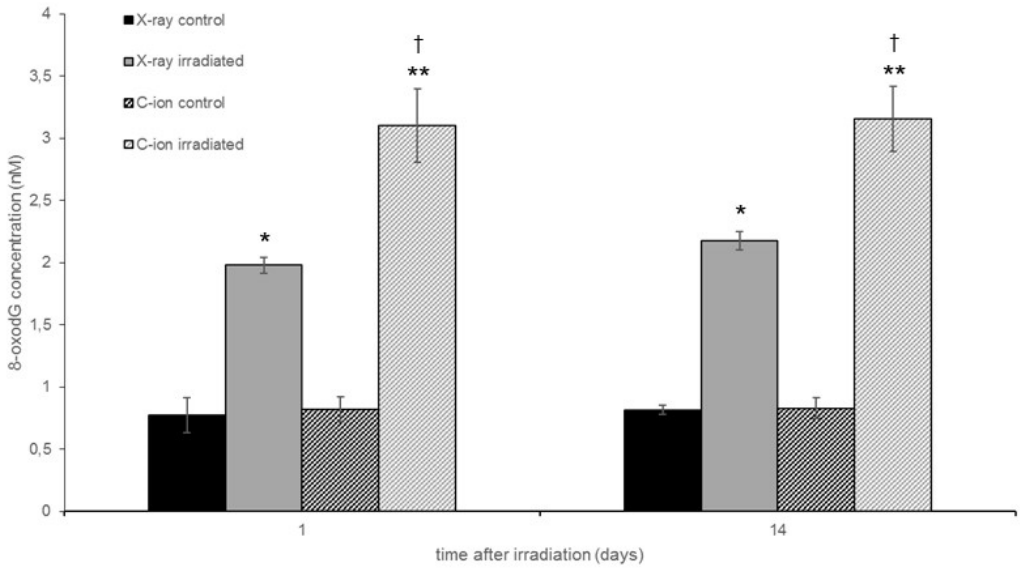

X-ray control
X-ray irradiated
C-ion control
C-ion irradiated
8-oxodG concentration (nM)
4
3,5
3
2,5
2
1,5
1
0,5
0
*
†
**
*
†
**
1
14
time after irradiation (days)

Figure 4

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