# Assessment of γH2AX nuclear foci number and size in normal and repair-deficient cells irradiated with low and high linear energy transfer radiation

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**Abstract:** The aim of this study was to evaluate the DNA damage induced by low and high linear energy transfer radiation in two related cell lines with different radiation sensitivity, CHO10B2 and irs-20 cells (defective in DNA-PKcs). Double-strand breaks were assessed by the detection of phosphorylated histone H2AX foci (γH2AX). The number of foci increased as a function of dose for both cell lines and types of radiation. However, irs-20 cells showed a higher number of foci than the parental CHO10B2. An increase in the foci size was observed for both cell lines after lithium irradiation. This could be attributed to clusters of DNA damage. Moreover, the number of lithium-induced larger foci per nuclei increased with dose and fitted with the expected number of hits per nucleus. Concluding, γH2AX foci size provides a potential tool to characterise the intrinsic radiosensitivity of cells related to DNA damage and to compare the effects of different quality radiations.

**Keywords:** ionising radiation; LET; linear energy transfer; DNA double strand break; γH2AX; H2AX foci size; DNA-PKcs; CHO10B2; irs-20.

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## 1 Introduction

DNA Double-Strand Breaks (DSBs) are probably the most dangerous of many different types of damage that occur within the cell, because they are highly mutagenic if misrepaired and lethal if left unrepaired (Ward, 1985). DSBs arise endogenously during DNA replication, V(D)J recombination, exchange meiotic, production of Reactive Oxygen Species (ROS) and are considered the most biologically damaging lesions generated by Ionising Radiation (IR) (Olive, 1998). IR also produces a broad spectrum of molecular lesions to DNA, including Single-Strand Breaks (SSBs), DSBs and a great variety of base damages (Van Gent, 2001).

The cellular responses to DSBs are a rapid and highly coordinated series of molecular events, resulting in DNA damage signalling and repair so-called DNA Damage Response (DDR) (Zhou and Elledge, 2000). This process is characterised by the accumulation

of repair proteins that generate domains, termed foci, which can be visualised by fluorescence microscopy (Petrini and Stracker, 2003). Eukaryotic cells repair DSBs primarily by two mechanisms: Non-Homologous End-Joining (NHEJ) and Homologous Recombination (HR). In particular, DSBs produced by IR can be repaired by either pathway. A key requirement for mammalian NHEJ is the kinase activity and phosphorylation of clusters of serine and threonine residues in DNA-PKcs, targeted by DNA-PKcs itself and ATM (Kurimasa et al., 1999; Chan et al., 2002) These processes are critical for cellular radioresistance and for NHEJ (Ding et al., 2003).

Phosphorylation of histone H2AX, called γH2AX, is one of the earliest events after DSBs, which results in extensive chromatin modification in the vicinity of the lesion triggering DNA damage signalling (Pilch et al., 2003). It is interesting to note that the nature of the DNA lesion determines which kinase phosphorylates H2AX. For example, ATM and DNA-PKcs redundantly phosphorylate H2AX in response to DSBs induced by IR or chemical agents such as bleomycine, while ATR is involved in the formation of γH2AX in response to UV radiation and replication stress (Ismail, 2007).

Histone H2AX is a variant of histone H2A family that constitutes 2%–25% of mammalian histone H2A pool. H2AX has a conserved COOH-terminal region that is target of post-translational phosphorylation at serine 139 in response to DSBs (Rogakou et al., 1998; Rogakou et al., 1999). The detection of histone γH2AX by fluorescence microscopy has been used as a measure of the formation and rejoining of DSB detected as distinct foci that most likely represent a single DSB. Moreover the analysis of IR-induced foci has provided important information on the molecular processes underlying the DDR. Regarding the number of γH2AX foci formed, it has been shown to be directly proportional to the number of DSBs (Rogakou et al., 1998). It is estimated that roughly 2000 γH2AX are formed per DSB. This is what makes its detection the most sensitive method to evaluate DSBs in cells in a dose range comparable with the dose per fraction used in radiotherapy (Ibañez et al., 2009). Therefore, γH2AX foci are now generally accepted as consistent and quantitative markers of DSBs, applicable even under conditions where only few DSBs are present (Rothkamm and Lobrich, 2003).

It is well established that the effects of high Linear Energy Transfer (LET) radiation on biological systems are more detrimental than those of low-LET radiation (Pathak et al., 2007), as clustered DNA damage with two or more lesions within the DNA helix may be induced by the high ionisation density in the individual track of a single particle (Sutherland et al., 2001). As a consequence, the biological damage produced by high-LET radiations is less repairable compared to that induced by low-LET radiations. Moreover, high-LET radiations are reported to have better physical dose distribution and higher biological efficacy in cell killing (Debus et al., 1998). These characteristic features make them useful in cancer therapy (Orecchia et al., 1998). Furthermore, the study of high-LET radiations may reveal fundamental mechanisms of their biological effects (Pathak et al., 2007).

Although the damaging effects of high doses of ionising radiation on living systems are well known, the biological consequences of low dose radiations are not fully understood. Brenner et al. (2003) refers to DSBs induced in a dose-dependent manner even at low doses. However, these conclusions were derived from linear extrapolation of data obtained from high-dose radiation. This linearity assumption is not necessarily the most conservative approach, and it is likely to result in an underestimation of some

radiation-induced damages and an overestimation of others. This would be due to the lack of an assay sensitive enough to detect a small number of DSBs as it is feasible to perform by the detection of γH2AX.

The aim of this study was to further understand the response to DSBs. Within this context, we investigated DNA damage induced by high-LET (accelerated $^{6}$Li ions) and low-LET radiations (gamma rays) in terms of the number and size of nuclear foci in related cells with different radiosensitivity.

## 2 Materials and methods

### *2.1 Cell line and cell culture*

The cell lines used in this work were CHO10B2 of hamster origin and its radiation-sensitive mutant cell line irs-20, kindly provided by Dr. M. Muhlmann (Department of Radiobiology, National Atomic Energy Commission, Argentina). Irs-20 cells showed milder radiosensitivity in all phases of the cell cycle (Lin et al., 1997). Irs-20 expresses a defective DNA-PKcs protein as a result of a mutation that causes the substitution of a lysine for a glutamic acid in the fourth residue from the C-terminus. One of the principal consequences of these mutations is that DNA-PKcs in irs-20 cell retains the ability to bind DNA, but is unable to function as a protein kinase (Priestley et al., 1998).

Cells were grown in RPMI-1640 medium supplemented with 10% foetal bovine serum, 50U/ml penicillin and 50 mg/ml streptomycin, at 37°C in a 5% $CO_2$ humidified atmosphere and were subcultured following standard procedures.

### *2.2 Irradiation experiments*

Cells were irradiated at 70% confluence with 0.5–3 Gy of gamma rays and 0.2–3 Gy of lithium beams. For gamma irradiations, a $^{137}$Cs source from an IBL 437C H-type irradiator, CIS Bio International, Schering SA, was used. For lithium irradiation, the medium was removed and the dish covers were replaced by 1.5 μm Mylar foils. The plates were set up in vertical position with the cell layers perpendicular to the incident horizontal beam. Immediately after irradiation, complete fresh medium was added. Control cells were sham irradiated. The lithium beams were produced by an electrostatic 20 MV vertical tandem accelerator (TANDAR accelerator, National Atomic Energy Commission, Argentina) as previously described (Schuff et al., 2002). The energies of the particles were 14 ± 1 MeV and the mean LET 135 ± 7 keV/μm. Dosimetry was performed using a dose-calibrated transmission ionisation chamber, calibrated with a dose-calibrated Capintec PS-033 thin window parallel plate ionisation chamber (International Atomic Energy Agency, 2000). This chamber was placed at exactly the same position as the cell culture plane and the Mylar cover of the dishes had the same thickness as the chamber window, ensuring that the incident energy on the cells matches that in the sensitive volume of the parallel plate ionisation chamber. The energy of the particles and mean LET values on the cells' midplane, calculated as previously described (Schuff et al., 2002), are shown in Table 1.

**Table 1** Irradiation energies. Mean LET values on the cells midplane for gamma rays and lithium beams

| | *Mean energy (MeV)* | *Mean LET (keV/μm)* |
|---|---|---|
| Gamma rays | 0.667 | 0.2 |
| Lithium ($^6$Li) | 14 ± 1 | 135 ± 7 |

### *2.3 Immunofluorescence and quantification of γH2AX*

After irradiation, cells were maintained at 37°C and 5% $CO_2$ for 30 minutes until 4% paraformaldehyde fixation. Cells were fixed for 15 minutes, washed with Phosphate Buffered Saline (PBS), permeabilised with 0.5% Triton X-100 in PBS during 15 minutes, washed and blocked with 5% FBS in PBS during 30 minutes. After blocking, cells were incubated overnight at 4°C with a monoclonal anti-γH2AX antibody (Upstate, Lake Placid, NY), 1:500 in PBS, washed and incubated with FITC-labelled secondary antibody (Sigma) in the dark for 1 h at room temperature. Cells were then washed, counterstained and mounted with 1 μg/ml 4',6-diamidino-2-phenylindole dihydrochloride dihydrate (DAPI) in an antifade solution, in the dark. Cells were examined in an Olympus BX51 epifluorescence microscope utilising immersion oil with a 100X (UPlanApo 100 X/1.35 oil) objective lens. For each treatment condition, FITC and DAPI images were serially captured by a CCD camera (Olympus DP70) and more than 50 fields containing approximately 20 cells each were stored. A code number was given to each image. Aleatory sampling methods were used to select the images and all the cells in each selected image were screened. An average of 150 cells was evaluated per each experimental condition. The γH2AX foci per nucleus were counted by eye by two scorers and results were crosschecked. The area of each γH2AX focus was quantified by using the NIH Image J software. Two independent experiments were performed with triplicates for each condition.

### *2.4 Statistical analysis*

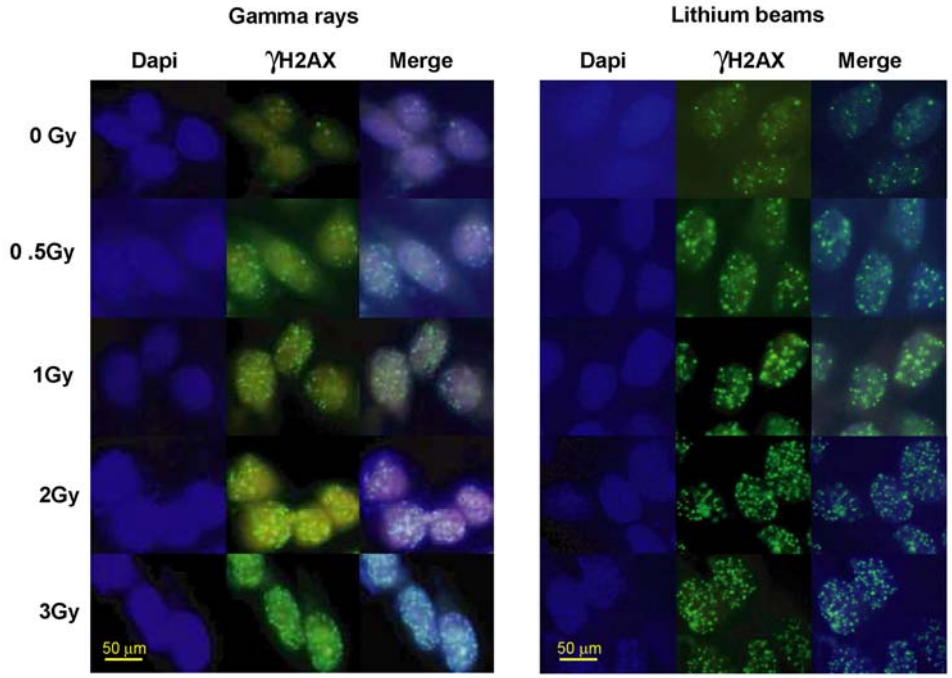
The results are presented as mean ± SD. Significant changes were assessed using one-way analysis of variance and non-parametric Kruskal–Wallis test followed by Tukey's or Dunn's multiple comparison tests to determine significant differences between group means. $p$ values of less than 0.05 were considered significant for all tests. Regression analysis was performed when appropriate.

## 3 Results

To evaluate the formation of DSBs, the number and size of nuclear γH2AX foci induced by gamma radiation and lithium particles in CHO10B2 and irs-20 cells were quantified. Images from a representative experiment of cells irradiated with gamma ray and lithium beams as a function of dose are shown in Figure 1. Initially, the number of

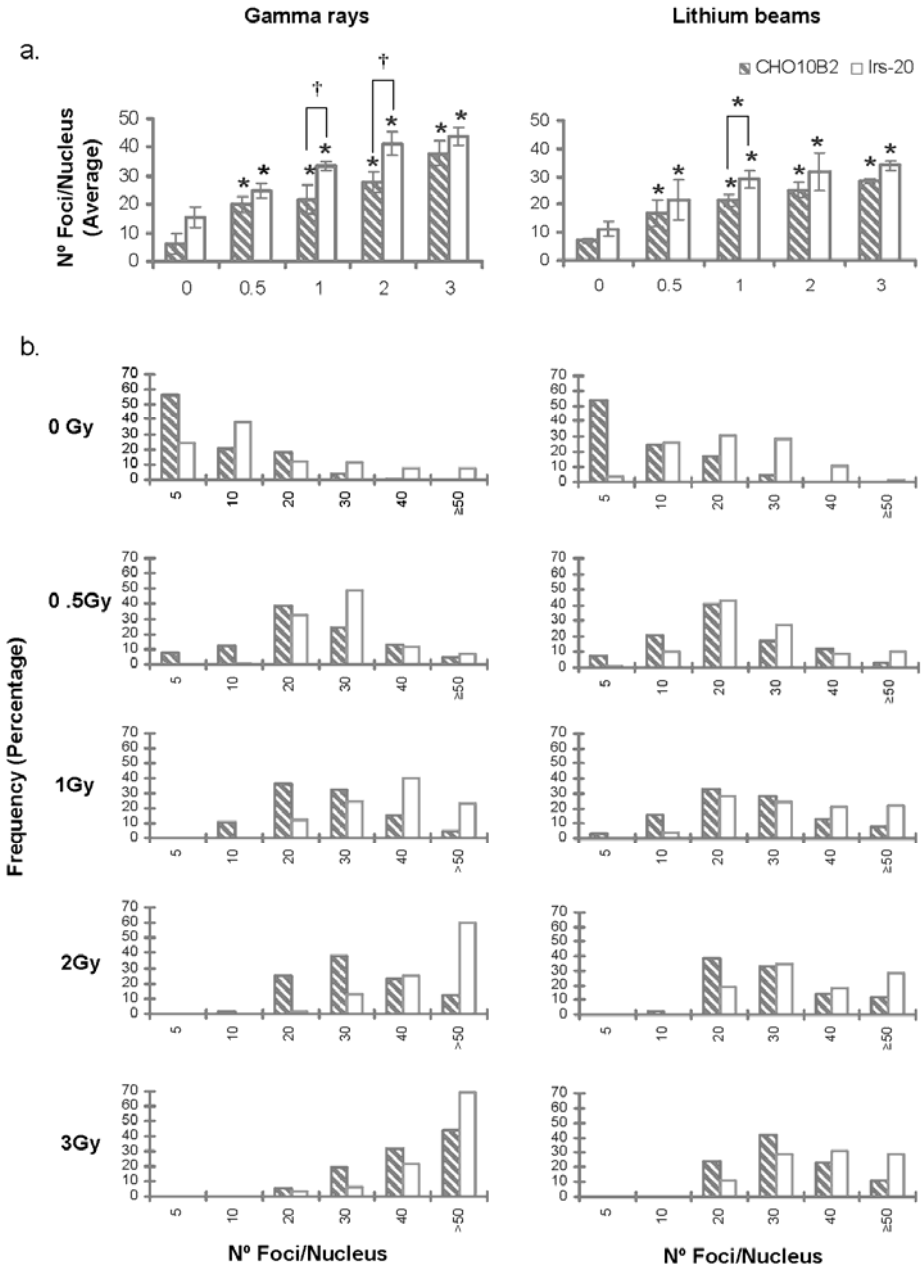
γH2AX foci per nucleus as a function of dose, with low and high-LET radiations, was quantified (Figure 2a). The number of foci was significantly ($p < 0.05$) higher than the corresponding non-irradiated control for all the experimental conditions. The comparison between both cell lines indicates higher values for irs-20, probably due to its greater radiosensitivity. Regarding the comparison between both types of radiation, no significant increase in the number of foci was found. The frequency histograms for gamma and lithium beams show a high number of nuclei with few foci at low doses and an increase in the number of foci per nucleus at higher doses (Figure 2b). Note that for higher doses almost none of the cells have less than 20 foci. For instance, after 2 Gy of gamma rays around 60% of CHO10B2 and only 17% of irs-20 cells present less than 40 foci per nucleus, while more than 80% of irs-20 show 40 foci per nucleus or more. This implies a higher number of DSBs at a given dose in the radiosensitive mutant irs-20. However, after lithium irradiation a remarkably high percentage of cells with a high number of foci per nucleus were detected for both cell lines.

**Figure 1** Representative images showing nuclear γH2AX foci (see online version for colours)




Notes: CHO10B2 and irs-20 cells exposed to 0.5–3 Gy of gamma rays and lithium beams. An increase in the number of foci/nucleus post-irradiation vs. unirradiated cells can be observed.
DAPI: marker of DNA that stains the whole nucleus of a cell.
γH2AX: detected with an anti-γH2AX antibody developed by a FITC conjugated secondary antibody.
Merge: overlapped images stained with DAPI and FITC conjugated.

**Figure 2** Quantification of nuclear γH2AX foci




Notes: CHO10B2 and irs-20 cells exposed to 0.5–3 Gy of gamma rays and lithium beams

(a) Average number of γH2AX foci/nucleus vs. dose.

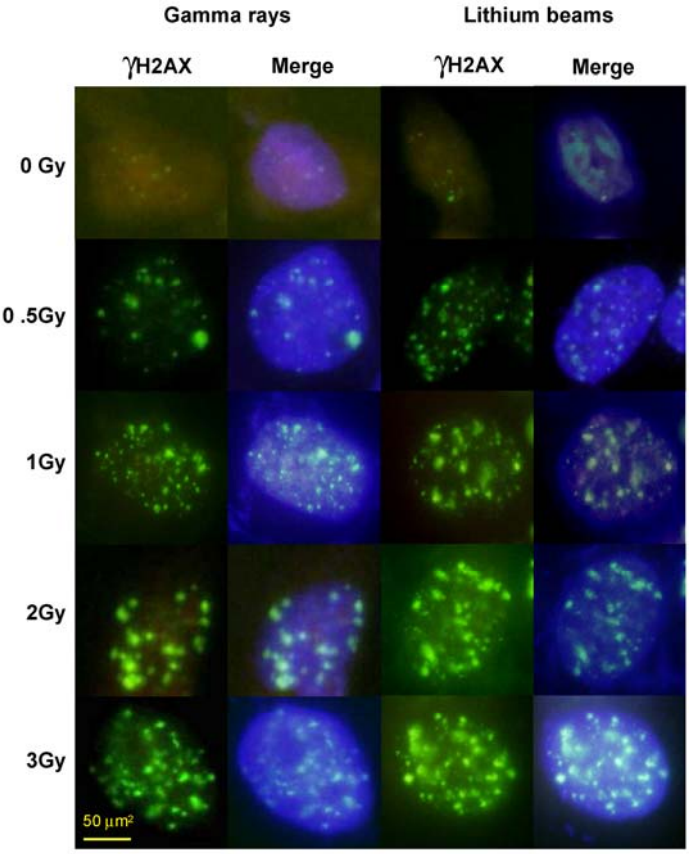
Data represent mean ± SD.

* $p < 0.01$ and † $p < 0.001$ vs. control or between cell lines.

(b) Frequency histograms of percentage of cells presenting 0 to more than 50 γH2AX foci/nucleus for different doses and types of radiation.

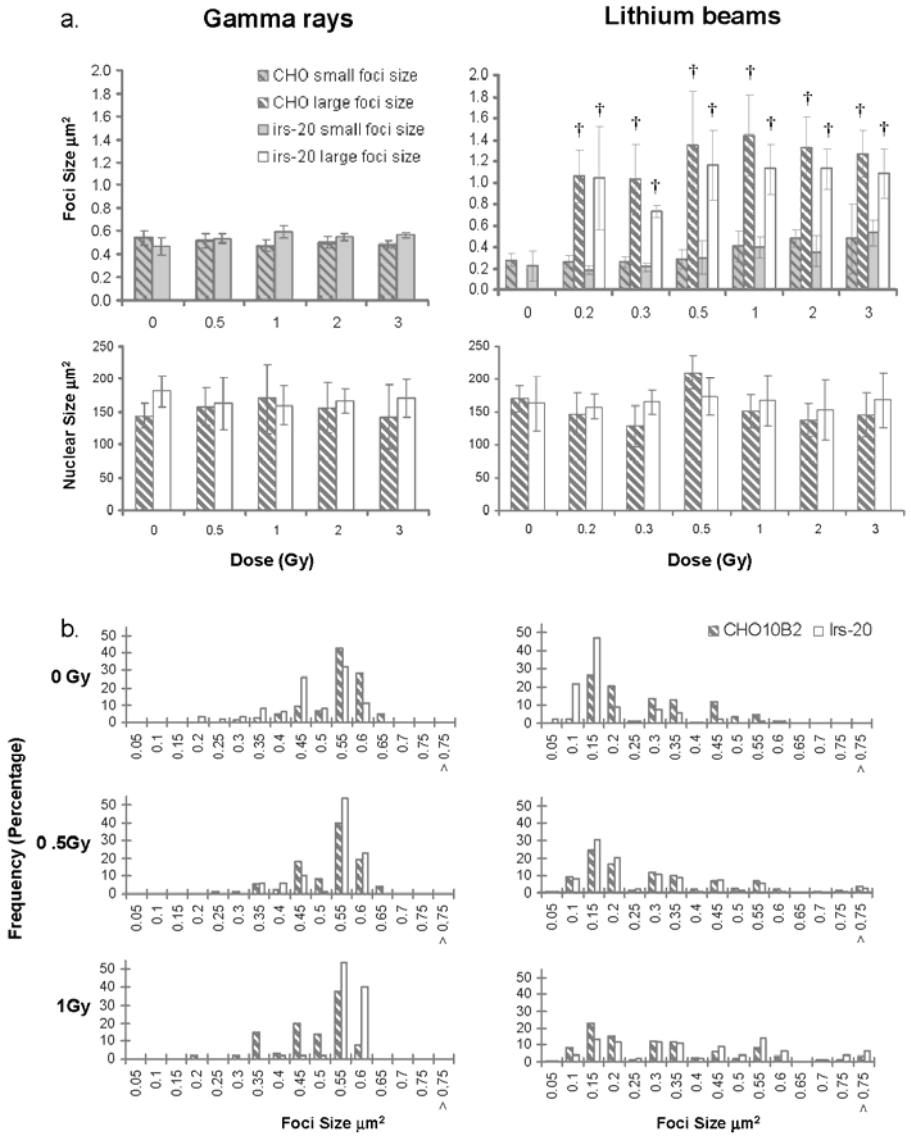
The microscopic observation of nuclear foci induced by IR revealed an increase in foci size after high-LET radiation. Variations in foci size and in the amount of large foci after gamma and lithium irradiation are shown in Figure 3. In view of these results, considering that DSBs induced by high-LET radiations are densely concentrated in clusters and the evident marked increase in the foci areas after all lithium doses, a quantitative analysis of the foci size was performed. A significant increase ($p < 0.01$) in foci size after lithium irradiations in comparison with cells exposed to gamma rays was demonstrated (Figure 4a). Moreover, the foci size induced by lithium beams is double that obtained in cells exposed to gamma rays, which is consistent with the highly dense DSBs in DNA clustered damage induced by high-LET radiation. Frequency histograms of foci size (Figure 4b) show that the foci areas in gamma-irradiated cells are always under 0.7 $\mu m^2$. On the other hand, after lithium irradiations, a clear shift towards larger foci is observed showing greater values as a function of dose.

**Figure 3** Representative images showing nuclear γH2AX foci size (see online version for colours)




Notes: CHO10B2 and irs-20 cells exposed to 0.5–3 Gy of low and high LET radiations. An increase in foci size after high LET radiation can be observed.
γH2AX: detected with an anti-γH2AX developed by a FITC conjugated secondary antibody.
Merge: overlapped images stained with DAPI and FITC conjugated.

**Figure 4** Quantification of nuclear γH2AX foci size

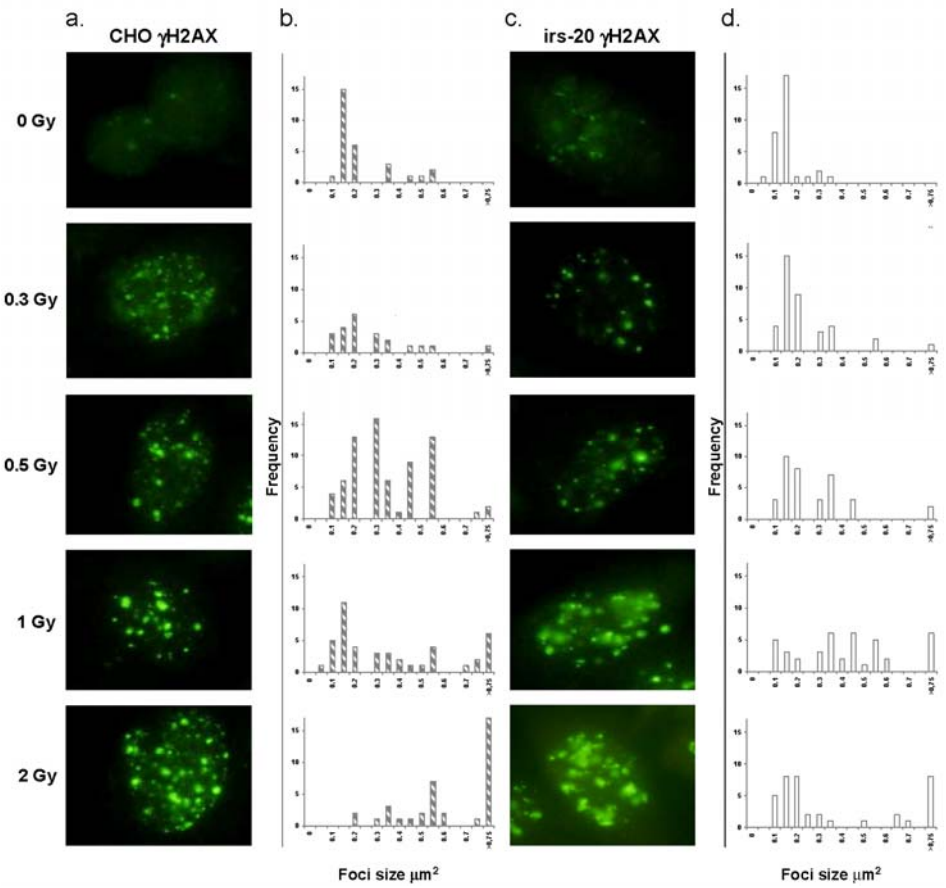



Notes: (a) γH2AX foci size (top) and nuclear size (bottom) vs. dose for CHO10B2 and irs-20 cells irradiated with 0.5–3 Gy of gamma rays and 0.2–3 Gy of lithium beams.
Data represent mean ± SD. † $p < 0.001$ vs. control.
(b) Frequency histograms of percentage of cells presenting 0 to more than 50 γH2AX foci per nucleus for different doses and types of radiation.

The relationship between the number of γH2AX foci induced by high-LET radiation and the number of hits per nucleus was evaluated. The actual particle fluence and the nuclear size were considered to calculate the average number of hits per nucleus. Typical fluences for 0.3–2 Gy are shown in Table 2. A striking correspondence between the

expected value of hits per nucleus and the number of foci larger than 0.7 $\mu m^2$ was found (Table 2). In order to illustrate the relationship between the number of large foci and fluence, images with the corresponding frequency histogram of CHO10B2 and irs-20 cells are shown in Figure 5. Note the higher number of large foci as the dose increased for both cell lines. The average number of large foci per nucleus corresponded to the expected average of hits per nucleus. Neighbouring smaller foci can also be visualised. Thus, these larger foci would be the direct result of the lithium particle track, seen as large clusters of DSBs. The smaller foci that increased the scored average number of foci could not be attributed to the primary particle track.

**Figure 5** Average number of large foci/nucleus and the expected number of hits/nucleus (see online version for colours)




Notes: (a, c) Representative images of nuclear γH2AX foci post-irradiation in CHO10B2 and irs-20 cells exposed to 0.3–2 Gy of lithium beams.
(b, d) Corresponding frequency histograms (foci sizes) for each cell line at different doses. The average number of the large foci per nucleus corresponded to the expected average number of hits/nucleus (shown in Table 2). Neighbouring smaller foci can also be visualised.

**Table 2** Particle fluence for 0.3–2 Gy of lithium beams, the calculated average number of hits per cell nucleus (hits/nucleus) and the number of γH2AX foci larger than 0.7 μm$^2$ per cell nucleus (γH2AX foci > 0.7 μm$^2$) in CHO10B2 and irs-20 cells

| *Dose (Gy)* | *Fluence (particles/100 μm$^2$)* | *Hits/nucleus* | | *γH2AX Foci >0.7 γm$^2$* | |
|---|---|---|---|---|---|
| | | *CHO10B2* | *irs-20* | *CHO10B2* | *irs-20* |
| 0.3 | 1.3 | 1.78 | 2.28 | 0.59 ± 0.12 | 0.26 ± 0.08 |
| 0.5 | 2.3 | 4.6 | 4 | 2.45 ± 0.45 | 2 ± 0.21 |
| 1 | 4.6 | 6.9 | 7.73 | 4.27 ± 0.38 | 4.29 ± 0.6 |
| 2 | 9.2 | 12.6 | 17.76 | 7.46 ± 0.76 | 8.1 ± 0.6 |

## 4 Discussion

High-LET radiations such as accelerated lithium particles lead to severe biological consequences due to the formation of clustered DNA damage (Terato and Ide, 2004). Thus, the characterisation of clustered DNA damage within CHO10B2 and irs-20 cells was performed to understand the effects of this type of damage. At the outset, we demonstrated an increase in the number of γH2AX foci as a function of dose for both types of radiation and cell lines assayed. The number of foci was higher for irs-20, presumably because of its defective DNA-PKcs, which is involved in DNA repair by NHEJ. This process requires the kinase activity and phosphorylation of DNA-PKcs, targeted by DNA-PKcs itself and ATM. Hence, this mutation renders irs-20 unable to repair DSBs by NHEJ (Chan et al., 2002). However, an increase in HR seen in cells with NHEJ defects is consistent with a passive shunting of DSBs repair from NHEJ to HR (Clikeman et al., 2001). Furthermore, several lines of evidence suggested that DNA-PKcs is an active regulator of DSB repair pathways choice (Allen et al., 2002). Shrivastav (2008) demonstrated that when DNA-PKcs null CHO V3 cells were complemented with a DNA-PKcs harbouring a single lysine to arginine change near the kinase active site, HR was stimulated threefold above the HR levels seen in the control cells. These results might explain those of our laboratory where irs-20 that expresses a defective DNA-PKcs resulting from the substitution of a lysine for a glutamic acid close to the C-terminus always showed a greater number of γH2AX foci than CHO10B2, probably due to a shift towards the HR pathway mainly responsible for the DSBs repair process.

Gamma rays and lithium particles generate DNA fragments with distinct sizes. Then, the fragment sizes resulting from DSBs may reflect both the track of the incident IR and the extent of the damage resulting from a particular type of radiation (Hiroaki and Hiroshi, 2004). The amount of H2AX molecules phosphorylated in response to a single DSB and its positioning within the cell nucleus in relation to the radiation tracks may influence the size of the foci observed (Leatherbarrow et al., 2006). Furthermore, each high-LET radiation track induces clusters of multiple DSBs along its core which cannot be resolved by fluorescence microscopy and for that reason could appear as a single focus (Costes et al., 2006). These limitations to resolve DSB induced by charged particles could explain the lack of increase in the number of foci described herein for high-LET vs. low-LET radiations. Thus, the fact that most of the reports have considered that one DSB generates one γH2AX focus, assuming the number of foci per nucleus independent of the foci size, must be reconsidered in cells exposed to high-LET radiation (Costes et al., 2006).

Hence, we measured the foci size and proposed the existence of two foci population: (a) small foci, with areas between 0.1–0.6 $\mu m^2$ that were observed after gamma and lithium irradiations with not significant differences between them or with control and (b) large foci (>0.7 $\mu m^2$) found only in cells exposed to lithium beams. These were significantly larger than the small foci. Control did not exhibit any larger foci. Besides, the formation of large foci was not associated with changes in nuclear area, which remained at approximately 150 $\mu m^2$.

Within this context, the particle track structure of lithium ions was taken into account and a correspondence between the expected number of hits per nucleus and the number of γH2AX foci with an area larger than 0.7 $\mu m^2$ was found. These results are in agreement with a previous study by our laboratory (Ibañez et al., 2009) where in a murine melanoma cell line a striking correspondence between large foci and the expected value of hits per nucleus after high-LET radiation was found. Thus, the larger foci would be the direct result of the lithium particle track and each large focus would represent a cluster of DNA and chromatin damage (Jakob et al., 2003). On the other hand, the smaller foci, that would be the result of either the induction of DNA damage by δ-rays, secondary nuclear fragments or possible bystander effects, contribute to the increase in the average number of foci per nucleus (Leatherbarrow et al., 2006).

Concluding, this study provides quantitative data on the induction of large γH2AX foci by high-LET radiation in related cell lines compared with those observed after low-LET radiation. A close relationship was detected between large foci number and particle fluence for all dose assayed. Thus, our results on γH2AX foci size contribute with a potential tool to characterise the intrinsic radiosensitivity of cells with differential genetic features related to DDR and to compare the effects of different quality radiations.

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## References

Allen, C., Kurimasa, A., Brenneman, M.A., Chen, D.J. and Nickoloff, J.A. (2002) 'DNA-dependent protein kinase suppresses double-strand break-induced and spontaneous homologous recombination', *Proceedings of National Academy of Sciences of the United States of America*, Vol. 99, No. 6, pp.3758–3763.

Brenner, D.J., Doll, R., Goodhead, D.T., Hall, E.J., Land, C.E., Litle, J.B., Lubin, J.H., Preston, D.L., Preston, R.J., Puskin, J.S., Ron, E., Sachs, R.K., Samet, J.M., Setlow, R.B. and Zaider, M. (2003) 'Cancer risk attributable to low doses of ionizing radiation: assessing what we really know', *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 100, No. 24, pp.4973–4975.

Chan, D.W., Chen, B.P., Prithivirasingh, S., Kurimasa, A., Story, M.D., Qin, J. and Chen, D.J. (2002) 'Autophosphorylation of the DNA-dependent protein kinase catalytic subunit is required for rejoining of DNA double-strand breaks', *Genes & Development*, Vol. 16, No. 18, pp.2333–2338.

Clikeman, J.A., Khalsa, G.J., Barton, S.L. and Nickoloff, J.A. (2001) 'Homologous recombinational repair of double-strand breaks in yeast is enhanced by MAT heterozygosity through yKU-dependent and independent mechanisms', *Genetics*, Vol. 157, No. 2, pp.579–589.

Costes, S.V., Boissiere, A., Ravani, S., Romano, R., Parvin, B. and Barcellos-Hoff, M.H. (2006) 'Imaging features that discriminate between foci induced by high- and low-LET radiation in human fibroblasts', *Radiation Research*, Vol. 165, No. 5, pp.505–515.

Debus, J., Jackel, O., Kraft, G. and Wannenmacher, M. (1998) 'Is there a role for heavy ion beam therapy?', *Recent Results in Cancer Research*, Vol. 150, pp.170–182.

Ding, Q., Reddy, Y.V., Wang, W., Woods, T., Douglas, P., Ramsden, D.A., Lees-Miller, S.P. and Meek, K. (2003) 'Autophosphorylation of the catalytic subunit of the DNA-dependent protein kinase is required for efficient end processing during DNA double-strand break repair', *Molecular and Cell Biology*, Vol. 23, No. 16, pp.5836–5848.

Hiroaki, T. and Hiroshi, I. (2004) 'Clustered DNA damage induced by heavy ions particles', *Biological Sciences in Space*, Vol. 18, No. 4, pp.206–215.

Ibañez, I.L., Bracalente, C., Molinari, B.L., Palmieri, M.A., Policastro, L., Kreiner, A.J., Burlon, A.A., Valda, A., Navalesi, D., Davidson, J., Davidson, M., Vazquez, M., Ozafran, M. and Duran, H. (2009) 'Induction and rejoining of DNA double strand breaks assessed by H2AX phosphorylation in melanoma cells irradiated with proton and lithium beams', *International Journal of Radiation Oncology, Biology, Physics*, Vol. 74, No. 4, pp.1226–1235.

International Atomic Energy Agency (2000) *Absorbed Dose Determination in External Beam Radiotherapy*, IAEA 2000 Technical Report Series No. 398, Vienna.

Ismail, I.H., Wadhra, T.I. and Hammarsten, O. (2007) 'An optimized method for detecting gamma-H2AX in blood cells reveals a significant interindividual variation in the gamma-H2AX response among humans', *Nucleic Acids Research*, Vol. 35, No. 5, p.e36.

Jakob, B., Scholz, M. and Taucher-Scholz, G. (2003) 'Biological imaging of heavy charged-particle tracks', *Radiation Research*, Vol. 159, No. 5, pp.676–684.

Kurimasa, A., Kumano, S., Boubnov, N.V., Story, M.D., Tung, C.S., Peterson, S.R. and Chen, D.J. (1999) 'Requirement for the kinase activity of human DNA-dependent protein kinase catalytic subunit in DNA strand break rejoining', *Molecular and Cell Biology*, Vol. 19, No. 5, pp.3877–3884.

Leatherbarrow, E.L., Harper, J.V., Cucinotta, F.A. and O'Neill, P. (2006) 'Induction and quantification of gamma-H2AX foci following low and high LET-irradiation', *International Journal of Radiation Biology*, Vol. 82, No. 2, pp.111–118.

Lin, J.Y., Muhlmann-Diaz, M.C., Stackhouse, M.A., Robinson, J.F., Taccioli, G.E., Chen, D.J. and Bedford, J.S. (1997) 'An ionizing radiation-sensitive CHO mutant cell line: irs-20. IV. Genetic complementation, V(D)J recombination and the scid phenotype', *Radiation Research*, Vol. 147, No. 2, pp.166–171.

Olive, P.I. (1998) 'The role of DNA single- and double-strand breaks in cell killing by ionizing radiation', *Radiation Research*, Vol. 150, No. 5, pp.S42–51.

Orecchia, R., Zurlo, A., Loasses, A., Krengli, M., Tosi, G., Zurrida, S., Zucali, P. and Veronesi, U. (1998) 'Particle beam therapy (hadrontherapy): basis for interest and clinical experience', *European Journal of Cancer*, Vol. 34, No. 4, pp.459–468.

Pathak, R., Sarma, A., Sengupta, B., Det, S.K. and Khuda-Bukhsh, A.R. (2007) 'Response to high LET radiation 12C (LET, 295 keV/microm) in M5 cells, a radio resistant cell strain derived from Chinese hamster V79 cells', *International Journal Radiation Biology*, Vol. 83, No. 1, pp.53–63.

Petrini, J.H. and Stracker, T.H. (2003) 'The cellular response to DNA double-strand breaks: defining the sensors and mediators', *Trends in Cell Biology*, Vol. 13, No. 8, pp.458–462.

Pilch, D.R., Sedelnikova, O.A., Redon, C., Celeste, A., Nussenzweing, A. and Bonner, W.M. (2003) 'Characteristics of gamma-H2AX foci at DNA double-strand breaks sites', *Biochemistry and Cell Biology*, Vol. 81, No. 3, pp.123–129.

Priestley, A., Beamish, H.J., Gell, D., Amatucci, A.G., Muhlmann-Diaz, M.C., Singleton, B.K., Smith, G.C., Blunt, T., Schalkwyk, L.C., Bedford, J.S., Jackson, S.P., Juego, P.A. and Taccioli, G.E. (1998) 'Molecular and biochemical characterisation of DNA-dependent protein kinase-defective rodent mutant irs-20', *Nucleic Acids Research*, Vol. 26, No. 8, pp.1965–1973.

Rogakou, E.P., Pilch, D.R., Orr, A.H., Ivanova, V.S. and Bonner, W.M. (1998) 'DNA double-strand breaks induce histone H2AX phophorilation on serine 139', *The Journal of Biological Chemistry*, Vol. 273, No. 10, pp.5858–5868.

Rogakou, E.P., Boon, C., Redon, C. and Bonner, W.M. (1999) 'Megabase chromatin domains involved in DNA double-strand breaks in vivo', *The Journal of Cell Biology*, Vol. 146, No. 5, pp.905–916.

Rothkamm, K. and Lobrich, M. (2003) 'Evidence of a lack of DNA double-strand break repair in human cells exposed to very low X-ray doses', *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 100, No. 9, pp.5057–5062.

Schuff, J.A., Policastro, L., Duran, H., Kreiner, A.J., Mazal, A., Molinari, B.L., Burlon, A., Debray, M.E., Kesque, J.M., Somacal, H., Stoliar, P., Valda, A., Bernaola, O.A., Perez de la Hoz, A., Saint-Martin, G., O'Connor, S., Davidson, J., Davidson, M., Naab, F., Ozafran, M.J., Vazquez, M.E., Caneva, S., Delacroix, S., Favaudon, F., Henry, Y., Nauraye, C, Brune, E., Gautier, C, Habrand, J.L., Palmieri, M. and Ruffolo, M. (2002) 'Relative biological effectiveness measurements of low energy protons and lithium beams on tumor cells', *Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms*, Vol. 187, No. 3, pp.345–353.

Shrivastav, M., De Haro, L.P. and Nickoloff, J.A. (2008) 'Regulation of DNA double-strand break repair pathway choice', *Cell Research*, Vol. 18, pp.134–147.

Sutherland, B.M., Bennett, P.V., Schenk, H., Sidorkina, O., Laval, J., Trunk, J., Monteleone, D. and Sutherland, J. (2001) 'Clustered DNA damages induced by high and low LET radiation, including heavy ions', *Physics Medicine*, Vol. 17, No. 1, pp.202–204.

Terato, H. and Ide, H. (2004) 'Clustered DNA damage induced by heavy ions particles', *Biological Science in Space*, Vol. 18, No. 4, pp.206–215.

Van Gent, D.C., Hoeijmakers, J.H. and Kanaar, R. (2001) 'Chromosomal stability and the DNA double-stranded break connection', *Nature reviews Genetics*, Vol. 2, No. 3, pp.196–206.

Ward, J.F. (1985) 'Biochemistry of DNA lesions', *Radiation Research*, Vol. 104 pp.S103–111.

Zhou, B.B. and Elledge, S.L. (2000) 'The DNA damage response: putting checkpoints in perspective', *Nature*, Vol. 408, No. 6811, pp.433–439.