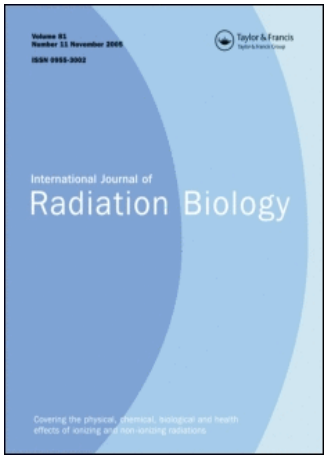


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# Genetic instability induced by low doses of x-rays in hamster cells

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**Abstract**

*Purpose:* Genomic instability involves time delayed events and can be manifested as elevated rates of heritable changes in the progeny of irradiated cells. To study the induction of chromosomal instability by very low doses of radiation Chinese Hamster Ovary (CHO) cells were exposed to 10 – 50 milisieverts (mSv) (≈10 – 50 miligrays (mGy)) of x-rays.

*Materials and methods:* Control and irradiated cell populations were assayed for chromosomal aberrations and assessed using a micronucleus test and anaphase-telophase analysis at the first cell division post-irradiation and at every four population doublings thereafter up to 16 population doublings post-irradiation.

*Results:* Frequencies of micronuclei, anaphase-telophase alterations and chromosomal aberrations were increased when the cells were analysed immediately after x-ray exposure. Micronuclei and anaphase-telophase alterations showed significantly increased frequencies when they were analysed at 12 and 16 population doublings after exposure to 50 mSv. Chromosomal aberrations increased significantly at 12 and 16 population doublings after exposure to 10 mSv and 50 mSv.

*Conclusions:* Our results are consistent with the presence of a phenomenon by which the initial DNA damage in the surviving cells is memorized. Micronuclei and achromatic lessions were the main cytogenetic damage observed in cells exposed to very low doses of x-rays, indicating that these low doses are able to induce genetic instability.

**Keywords:** *Genetic instability, ionizing radiation, low doses*

## Introduction

It is widely accepted that ionizing radiation is a mutagenic agent capable of inducing deleterious effects in human beings. The biological effects of exposure to radiation were considered for many years to be a consequence of direct DNA damage that was not correctly restored by metabolic repair processes. Although it is assumed that the risk of genotoxic damage increases in proportion to the dose of radiation, the situation is much less clear at low doses (Brenner et al. 2003). Recently, several lines of evidence have suggested new pathways for radiation-induced genetic damage, mainly after low dose exposure (Little 2003, Morgan 2003). Two non-targeted phenomena, genomic instability and the bystander effect, have been described over the last two decades suggesting that ionizing radiation exposure may induce damage in cells that are not being directly irradiated. Genomic instability involves time delayed events whereas the bystander effect includes the occurrence of damage in cells that are not themselves directly transversed by radiation.

Genomic instability can be manifested as elevated rates of heritable changes in the progeny of irradiated cells. It can be measured as chromosomal aberrations, micronucleus formation, gene mutations and microsatellite instabilities as well as other end points, but chromosomal changes are the best described (Morgan 2003, Limoli et al. 2000). Stable aberrations can be transmitted through many generations of cell replication (Little 2003). They can appear *de novo* in the progeny of cells that survive the original radiation exposure (Smith et al. 2003). A great variety of papers have reported the induction of chromosomal instability by low dose radiation exposure. Different cellular types such as lymphocytes, HPV-G transfected keratinocytes, CHO and other cells showed evidence of delayed chromosomal damage after exposure to $\gamma$- or $\chi$-rays (0.1 – 12 Grays) (Gy) or $\alpha$-particles

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(Holmberg et al. 1998, Bartolleto et al. 2001, Mothersill et al. 2000, Little et al. 1997, Ponnaiya et al. 2004). Elevated rates of micronucleated cells were observed in the progeny of primary human fibroblasts and V-79 cells irradiated with $\chi$ rays or $\alpha$-particles (Belyakov et al. 1999, Trott et al. 1998, Jamali & Trott 1996). On the other hand, no evidence of persistent transmissible genomic instability was found in a study of blood lymphocytes in the bone marrow of radiation workers with internal deposits of plutonium (Whitehouse & Tawn 2001) or in normal diploid human fibroblasts (AG1521A) surviving after exposure in G(0) to low- and high-LET radiation (Dugan & Bedford 2003).

In order to establish the induction of chromosomal instability by very low doses of radiation CHO cells were exposed to 10–50 milisievert (mSv) ($\approx$10–50 mGy) of $\chi$-rays. Chromosomal aberrations and micronuclei were analysed at various times after the initial radiation exposure.

## Material and methods

### *Cells*

CHO cells obtained from the American Type Culture Collection (Manassas, VA, USA) were cultured in Ham's F10 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (Notocor Laboratories, Province of Cordoba, Argentina) and antibiotics (50 IU penicillin and 50 $\mu$g/ml streptomycin) (Bagó Laboratories, Buenos Aires, Argentina) in a humidified atmosphere with 5% $CO_2$. Cells were cultured in Falcon T-25 flasks (Nunc, Nalge Nunc International, Denmark) with 10 ml culture medium.

### *Experimental design*

Irradiation treatments were performed when the cells were 90–95% confluent. Two radiation doses were employed: 10 and 50 mSv, based on previous work in our laboratory (Güerci et al. 2003, 2004) and the dosimetry reported in epidemiological studies of human exposures to ionizing radiation (Barquinero et al. 1993, Paz y Miño et al. 1995, Balakrishnan & Rao 1999, Cardoso et al. 2001, Cavallo et al. 2002). The x-ray apparatus used was from Dental San Justo company (Buenos Aires, Argentina) operated at 65 kV and 5 mA. Doses were determined by using a Keithley Digital 35617 EBS microchamber PTW N 2336/414 dosimeter (C-Com Industries, Robertville, MO, USA). The dose rate employed was 50 mSv/min. Radiation was given from above through the medium and exposure times were 12 and 60 sec for 10 and 50 mSv respectively. For the irradiation treatment 10 ml of fresh medium was placed on the attached cells to avoid the presence of detached cells.

After treatment, cells were trypsinized, resuspended and divided into two fractions. One fraction was cultured for cytogenetic analysis and the other fraction continued in culture for 16 passages. A control group remained unirradiated.

The doubling time of CHO cells under these culture conditions was periodically checked in the laboratory using a bromodeoxyuridine technique (BrdU) and it varie between 12 and 15 h (Grillo & Dulout, 1995) for both control and irradiated cells.

Control and irradiated populations were assayed for chromosomal aberrations, frequency of micronuclei and anaphase-telophase analysis at the first cell division post-irradiation, and at every four population doublings thereafter up to 16 population doublings post-irradiation. Each experiment was repeated twice and mean values are shown in Tables I–V.

### *Cytogenetic analysis*

Cells were cultured as monolayers for 15–16 hr. Two hours before fixation colchicine (Merk, Darmstadt, Germany), 1 $\mu$g/ml final concentration, was added to the cultures. Cells were then removed from each flask by trypsinization and agitation. The cell suspension was centrifuged and the pellet resuspended in 5 ml of hypotonic solution (KCl 0.075 M) for 20–22 min at 37°C. One ml of fixative (methanol:acetic acid 3:1) was added to the suspension before the cells were pelleted and resuspended in fixative. Cells were dropped onto clean slides and stained with 4% Giemsa for 10 min.

Table I. Mean frequencies (average $\pm$ standard error) of micronuclei, structural chromosome aberrations and anaphase-telophase alterations in irradiated and non-irradiated CHO cells. Analysis performed immediately after irradiation.

| | | Chromosomal aberrations (%) | | | | |
|---|---|---|---|---|---|---|
| Treatment | Micronuclei (‰) | Achromatic lesions | Chromatid breaks | Isochromatid breaks | Abnormal cells | Anaphase-telophase alterations (%) |
| – | 4.0 $\pm$ 0.06 | – | – | – | – | 0.5 $\pm$ 0.07 |
| 10 mSv | 11.0 $\pm$ 0.10 | 2.0 $\pm$ 0.14 | 1.0 $\pm$ 0.09 | – | 3.0 $\pm$ 0.17 | 2.0 $\pm$ 0.14 |
| 50 mSv | 9.0 $\pm$ 0.09 | 7.0 $\pm$ 0.25 | 2.0 $\pm$ 0.14 | – | 9.0 $\pm$ 0.28 | 4.0 $\pm$ 0.19 |

Table II. Mean frequencies (average ± standard error) of micronuclei, structural chromosome aberrations and anaphase-telophase alterations in irradiated and non-irradiated CHO cells. Analysis performed 4 cycles after irradiation.

| | | Chromosomal aberrations (%) | | | | |
|---|---|---|---|---|---|---|
| Treatment | Micronuclei (‰) | Achromatic lesions | Chromatid breaks | Isochromatid breaks | Abnormal cells | Anaphase-telophase alterations (%) |
| – | 5.0 ± 0.07 | 1.0 ± 0.09 | 1.0 ± 0.09 | – | 2.0 ± 0.14 | 1.0 ± 0.09 |
| 10 mSv | 5.0 ± 0.07 | 0.5 ± 0.14 | 0.5 ± 0.14 | – | 1.0 ± 0.09 | 1.0 ± 0.09 |
| 50 mSv | 5.0 ± 0.07 | 2.5 ± 0.15 | 1.0 ± 0.09 | – | 3.5 ± 0.18 | 1.5 ± 0.12 |

Table III. Mean frequencies (average ± standard error) of micronuclei, structural chromosome aberrations and anaphase-telophase alterations in irradiated and non-irradiated CHO cells. Analysis performed 8 cycles after irradiation.

| | | Chromosomal aberrations (%) | | | | |
|---|---|---|---|---|---|---|
| Treatment | Micronuclei (‰) | Achromatic lesions | Chromatid breaks | Isochromatid breaks | Abnormal cells | Anaphase-telophase alterations (%) |
| – | 4 ± 0.06 | 0.5 ± 0.07 | 0.5 ± 0.07 | – | 1.0 ± 0.09 | 0.5 ± 0.07 |
| 10 mSv | 5 ± 0.07 | 1.5 ± 0.12 | 0.5 ± 0.07 | – | 2.0 ± 0.14 | 2.0 ± 0.14 |
| 50 mSv | 10 ± 0.09 | 2.5 ± 0.15 | 1.0 ± 0.09 | – | 3.5 ± 0.18 | 3.5 ± 0.15 |

Table IV. Mean frequencies (average ± standard error) of micronuclei, structural chromosome aberrations and anaphase-telophase alterations in irradiated and non-irradiated CHO cells. Analysis performed 12 cycles after irradiation.

| | | Chromosomal aberrations (%) | | | | |
|---|---|---|---|---|---|---|
| Treatment | Micronuclei (‰) | Achromatic lesions | Chromatid breaks | Isochromatid breaks | Abnormal cells | Anaphase-telophase alterations (%) |
| – | 4 ± 0.06 | 2.0 ± 0.14 | – | – | 2.0 ± 0.14 | 1.0 ± 0.09 |
| 10 mSv | 11 ± 0.10 | 9.0 ± 0.28 | – | – | 9.0 ± 0.28 | 3.5 ± 0.18 |
| 50 mSv | 24 ± 0.15 | 14.0 ± 0.34 | 1.0 ± 0.09 | 1.0 ± 0.09 | 16.0 ± 0.36 | 5.0 ± 0.21 |

Table V. Mean frequencies (average ± standard error) of micronuclei, structural chromosome aberrations and anaphase-telophase alterations in irradiated and non-irradiated CHO cells. Analysis performed 16 cycles after irradiation.

| | | Chromosomal aberrations (%) | | | | |
|---|---|---|---|---|---|---|
| Treatment | Micronuclei (‰) | Achromatic lesions | Chromatid breaks | Isochromatid breaks | Abnormal cells | Anaphase-telophase alterations (%) |
| – | 5 ± 0.07 | 0.5 ± 0.07 | – | – | 0.5 ± 0.07 | 1.5 ± 0.12 |
| 10 mSv | 8 ± 0.08 | 3.0 ± 0.17 | 1.0 ± 0.09 | – | 4.0 ± 0.19 | 2.5 ± 0.15 |
| 50 mSv | 18 ± 0.13 | 7.0 ± 0.25 | 2.0 ± 0.14 | 1.0 ± 0.09 | 10.0 ± 0.30 | 5.0 ± 0.21 |

At least 200 metaphases per experimental point were scored for cytogenetic aberrations under light microscopy and classified following the criteria recommended by Archer and co-workers (Archer et al. 1981) and WHO (World Health Organization 1985). Non-staining or very lightly stained chromosome regions in one or both chromatids were considered as achromatic lesions (gaps) when there was no displacement of the chromatid fragment(s) distal to the lesion. If there was a displacement of the distal chromatid fragment or the non-staining region was wider than the chromatid width, the aberration was scored as a break.

Data for aberration frequencies in irradiated and control cells were statistically analyzed using the $\chi^2$-test.

### *Micronucleus test*

The cytokinesis-blocked micronucleus assay was modified from Fenech and Morley (1985). Cells were cultured as monolayers for 30 h. About 16 h

before harvesting $\beta$-cytochalasin (3 $\mu$g/ml final concentration) (Sigma, St. Louis, MO, USA) was added. Cells were then removed by trypsinization and agitation. The cell suspension was centrifuged and the pellet resuspended in 5 ml of fixative (methanol:acetic acid 3:1), the cells were washed with fresh fixative for three times, resuspended, dropped onto clean slides and stained with 4% Giemsa for 10 min. One-thousand binucleated cells were analysed per experimental point. Fenech and coworkers (Fenech et al. 2003) scoring criteria for micronuclei determinations were used. The $\chi^2$-test was used for statistical analysis.

*Anaphase-telophase analysis*

Cells were cultured as monolayers in 24 × 36 mm cover-glasses attached with a small drop of siliconized grease (Merk, Darmstadt, Germany) to the bottom of 90 mm Petri dishes, as was described in a previous work (Seoane & Dulout 1994). Cell harvesting was accomplished by adding an equal volume of fixative (methanol-acetic acid 3:1) to the culture medium. After 10 min two changes of fixative were made. Cover-glasses were stained with carbol fuchsin and attached with DPX mounting medium (Sigma, St. Louis, MO, USA) to coded slides. The cytogenetic alterations analysed were chromatin bridges, chromosomal fragments and lagging chromosomes. At least 200 anaphase-telophase images were observed per experimental point. The Sokal and Rohlf 'G' method (Sokal & Rohlf 1979) was used in order to compare the treatment conditions with the negative control.

## Results

To assay for the appearance of delayed cytogenetic alterations, irradiated populations of CHO cells were examined at every four population doublings up to 16 population doublings post irradiation (Figures 1–3). As expected, frequencies of micronuclei, anaphase-telophase alterations and chromosomal aberrations were increased compared to control cells, when the cells were analysed immediately after x-ray exposure (Table I). Chromosomal aberration frequencies were significantly different to that of controls at 10 and 50 mSv exposure ($p < 0.01$ and $p < 0.001$ respectively). Anaphase-telophase alteration frequencies only showed statistically significant differences when cells were exposed to 50 mSv ($p < 0.01$). No significant increases were observed for other frequencies.

Tables II–V show micronuclei, structural chromosomal aberrations and anaphase-telophase alterations percentages analysed after 4, 8, 12 and 16 population doublings respectively. Very small statistically insignificant increases were observed at the three end points when the cells were analysed after four and eight population doublings for

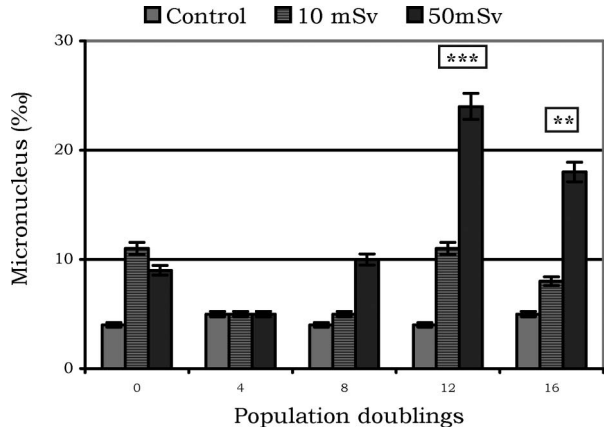


Figure 1. Micronuclei in CHO cells analysed immediately after irradiation and from 4 to 16 population doublings post irradiation. Error bars indicate average ± standard error. **Moderately significant; ***highly significant.

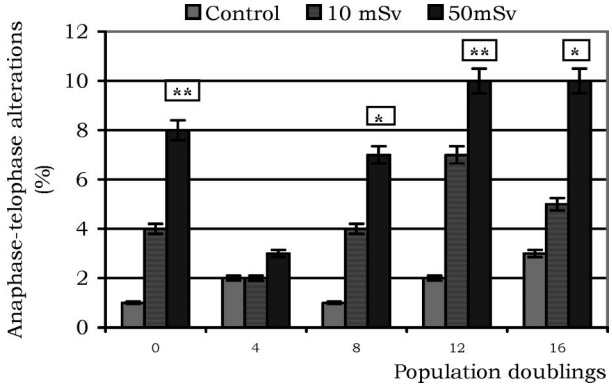


Figure 2. Anaphase-telophase alterations in CHO cells analysed immediately after exposition and from 4 to 16 population doublings post irradiation. Error bars indicate average ± standard error. **Moderately significant; ***highly significant.

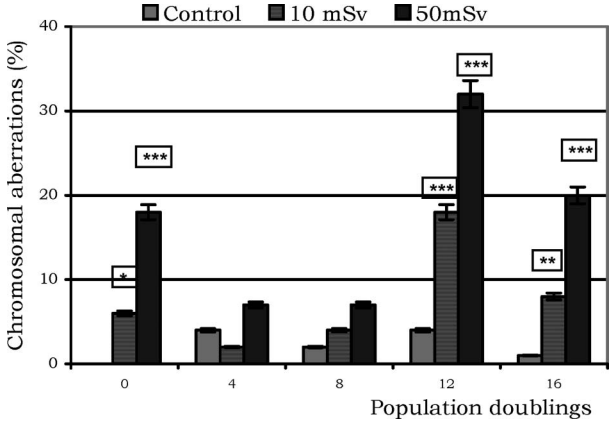


Figure 3. Chromosomal aberrations in CHO cells analysed immediately after exposition and from 4 to 16 population doublings post irradiation. Error bars indicate average ± standard error. **Moderately significant; ***highly significant.

exposure to 10 and 50 mSv. Only anaphase-telophase alterations showed significant differences after eight population doublings ($p < 0.05$).

Micronuclei showed significantly increased frequencies when they were analysed at 12 and 16 population doublings after exposure to 50 mSv ($p < 0.001$ and $p < 0.01$ respectively). A similar situation was observed for anaphase-telophase analysis ($p < 0.01$ and $p < 0.05$ respectively). Chromosomal aberrations increased significantly at 12 and 16 population doublings after exposure to 10 mSv ($p < 0.001$ and $p < 0.01$ respectively) and 50 mSv ($p < 0.001$).

No correlation analysis could be performed because only two x-rays doses were used. However, alteration frequencies were higher in cells exposed to 50 mSv than those exposed to 10 mSv for all end points analysed (Figures 1 – 3).

Likewise, frequencies of micronucleus, anaphase-telophase alterations and chromosomal aberrations were higher at 12 than at 16 population doublings.

## Discussion

Ionizing radiation is a physical agent that induces single and double strand breaks, base damage and DNA-protein crosslinks. Generally it is agreed that the most important lesion for the genetic risk of ionizing radiation exposure is the double strand breaks (DSB) which when not repaired or misrepaired can lead to genetic changes. The consequences of the induction of DSB can be observed cytogenetically at the first mitosis immediately following exposure to ionizing radiation as translocations, ring chromosomes, dicentrics, gaps and double minutes (Smith et al. 2003). In the present work only gaps or breaks were observed and no increase in chromosome type aberrations was detected at the first mitosis after exposure. A similar situation was observed at delayed times. Clastogenic events expressed as micronuclei, anaphase chromosome fragments and subchromatid or chromatid type aberrations could be scored.

Achromatic lesions have been the subject of controversy for years since their significance is not clear. Brecher (1977) has suggested that, rather than being completely separate phenomena, gaps and breaks are different manifestations of the same events and that gaps may be incomplete breaks. In this sense Dulout and coworkers (1983, 1985) have reported that gaps are transformed into breaks by blockage of the G2 check point by caffeine. Recent studies showed significant increases of gaps after low dose x-ray exposure (Güerci et al. 2003). In addition, similar results were found in air crew members (Cavallo et al. 2002) and hospital workers (Hagelstrom et al. 1995, Paz y Miño et al. 1995, Güerci 2004). These studies all agree in attributing the increase of gaps or chromatid type aberrations to chronic exposure to low level ionizing radiation.

Micronuclei originate from chromosome breaks or lagging chromosomes. Results described in the present work show that immediately after irradiation as well as after 12 and 16 population doublings an increase of micronuclei, anaphase-telophase alterations (lagging fragments) and achromatic lesions (gaps) could be observed. This fact is a clear indication that a number of the gaps are true chromatid or chromosome breaks and that the biological signification of gaps should be revaluated.

On the other hand, genomic instability can be manifested as stable aberrations transmitted through many generations of cell replication such as chromosomal reduplication, translocations or small deletions (Little 2003). In addition, it can be characterized by the *de novo* appearance of chromosomal damage in the progeny of cells that survived the original radiation exposure (Smith et al. 2003, Belyakov et al. 1999). Our results are consistent with these last observations because cytogenetic aberrations and micronuclei were observed immediately after exposure and after 12 replication cycles, but not (or not significantly) at replication cycles four to eight.

Although we are unable to establish a mechanism for these effects, our results agree with the presence of a phenomenon by which the initial DNA damage in the surviving cells is made permanent or 'memorized'. In this way Little (2003) hypothesized that radiation may induce changes affecting the genes directly and/or affecting either chromosomes or the cytoplasm in such a way as to increase the instability of the gene system. Susuki and coworkers (2003) have suggested that radiation exposure causes non-lethal, potentially unstable chromosome regions that are transmissible for many generations after irradiation. These unstable regions could cause delayed DNA breakage.

Numerous studies have established the occurrence of radiation-induced chromosomal instability in various cells types (Holmberg et al. 1998, Mothersill et al. 2000, Little et al. 1997, Limoli et al. 1999, Trott et al. 1998, Lorimore et al. 1998). In most of these studies, the cells were exposed to higher doses than those used in our experiments. Micronucleus induction at delayed times was reported for doses higher than those used in this work (Jamali & Trott 1996, Trott et al. 1998, Belyakov et al. 1999). Results described could reflect a complex response where very low doses of x-rays induced mutationally unstable cell survival instead of cell death. In this sense, Mothersill and Seymour (2003) support the idea that affected cells can die or live with damage and recover badly or well. If the cell does not recover well, it can die or perpetuate the damage.

In conclusion, cytogenetic damage could be observed in the progeny of cells exposed to very low doses of x-rays. Our results are consistent with the idea that low doses and low dose rates of x-rays may induce genomic instability. However, further studies are necessary to confirm these ideas.



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We wish to dedicate this work to the memory of Fernando N. Dulout who has recently passed away.

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