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EVALUATION OF RADIOADAPTIVE RESPONSE INDUCED IN CHO-K1 CELLS IN A NON TRADITIONAL MODEL

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Running head: Radioadaptive response in a non traditional model

Keywords: Radioadaptive response, structural chromosome aberration test, comet assay

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Abstract

Purpose: The present study was designed in order to evaluate sequential exposure to low doses of gamma-radiation that induce a radioadaptive response to a later high-dose radiation in CHO-K1 cells.

Materials and methods: Cells were cultured in 4 dilution cycles and grown to confluency. Radiation treatment was performed once per cycle with 0.1Gy gamma-rays. After the last radiation period (chronic radiation) the culture was irradiated with a higher dose (1Gy). Each cell culture was immediately divided into two fractions: one of them was used to carry out the comet assay and the other for the structural chromosome aberration test. In the first fraction, genotoxic damage was evaluated by degree of damage in 300 cells per experimental point. The second assay was performed in 400 cells per treatment. The statistical analysis was carried out using the χ^2 test.

Results: Results from these assays confirmed the genotoxic effect for both the adaptive and acute treatments ($p<0.001$). The comet assay showed a significant damage increase for the combined treatment when compared with 1Gy treatment ($p<0.001$). The frequency of chromosomal aberrations (CA) was lower for the combined treatment than for that using the highest radiation dose.

Conclusions: These results suggest the possible induction of a radioadaptive response after the sequential exposure to very low doses of radiation. The finding of cytogenetic damage decrease after one cellular cycle and not immediately after radiation could indicate the eventual potentiation of repair mechanisms.

Introduction

The understanding of the biological effects of ionizing radiation is essential for the elucidation of cellular response mechanisms and the assessment of risks from low-dose exposure.

So far, the biological effects of low-dose exposure have been estimated extrapolating data from high-dose radiation experiments, using a linear non-threshold (LNT) model.

Several changes have taken place in radiobiology over the last years. Recently, cellular and molecular studies on low-dose radiation have reported different phenomena such as

bystander effects, adaptive response induction, and genome instability (Azzam, *et al.,* 1998; Sawant *et al*., 2001, Venkat *et al*., 2001, Ballarini *et al*., 2002, Little *et al.,* 2002, Preston, 2004, Streffer, 2004 a-b).

The development of late effects following radiation exposure takes place through multiple steps that can involve gene mutations and chromosome aberrations, altered gene expression, and even changes in cell proliferation rates. During this multi-step process, induction of the adaptive response could lead to reduced effect degrees while the induction of genomic instability or the presence of bystander effects could promote late effects.

Adaptive response is defined as the development of resistance to a radiation-induced effect following a previous low-dose exposure (Samson and Cairns, 1977, Shadley *et al*., 1987, Wolff, 1998, Sasaki *et at.,* 2002). This phenomenon has been reported by many researchers for same organisms. The adaptive response was originally observed in human lymphocytes CA (Olivieri, *et al*., 1984). Later, it was described for occupationally exposed individuals (Barquinero *et. al*, 1995, Gourabi and Mozdarani 1998), cultured human lymphocytes (Wiencke *et al.,* 1986, Wolff *et al.,* 1988, Shadley and Wiencke 1989, Sankaranaryanan *et al,* 1989, Stoilov 2007), non-human lymphocytes (Flores *et al.,* 1996), cell lines (Ikushima 1987, Cortes *et al.,* 1990, Ishii and Watanabe 1996), insects (Fritz-Niggli and Schaeppi-Buechi 1991), and laboratory animals (Wojcik and Tuschl 1990, Cai and Liu 1990, Farooqi and Kesavan 1993). On the other hand, some reports have shown lack of radioadaptive response for cultured human lymphocytes (Bosi and Olivieri 1989, Hain *et al.,* 1992).

In vitro experimental models for low-dose exposure to ionizing radiation have been supported by experiments with only one adapting dose. However, many human individuals are chronically exposed to low doses of ionizing radiation (Carrano and Natarajan 1988, Au 1991). Thus, we have developed an *in vitro* test model in order to simulate a low-dose chronic exposure to gamma rays by means of cell cultures previously exposed to more than one adaptive dose, then irradiated with a higher dose.

Purpose

The present study was carried out to evaluate whether the sequential exposure of a Chinese hamster ovary cell line to low doses of gamma radiation induced a radio-adaptive response to a later high-dose radiation.

Materials and Methods

Cell cultures and experimental procedure

Chinese hamster ovary (CHO-K1) cell line was originally obtained from American Type Culture Collection (ATCC). Cells were grown as monolayer in Falcon T-25 flasks with 10 ml Ham F10 medium (GIBCO-BRL, Los Angeles, USA) supplemented with 10% inactivated fetal calf serum (Natocor, Córdoba, Argentina), 50 IU/ml penicillin, and 50 µg/ml streptomycin sulfate at 37º C in a 5% CO_2-humid atmosphere. Cell viability was checked using the trypan blue dye exclusion method; for all cases viability was higher than 90%.

Cells were cultured during 4 dilution cycles and grown to confluency. Radiation with 10mGy gamma rays was performed once per cycle when cells were at quiescent state. For all the experiments, cells were washed twice with phosphate-buffered saline (PBS), and irradiated in this solution at room temperature. After treatment, cells were trypsinized and resuspended with fresh medium. At each point of the serial procedure, the culture was diluted 1:2 to follow with chronic radiation. A little aliquot from the first and fourth radiation cycles was extracted in order to carry out the comet assay. After the last chronic radiation cycle the culture was divided into two fractions. One of them was irradiated with a high dose (1Gy) of gamma rays, and the other fraction was used to analyze the low-dose chronic effect. Each of the two fractions was divided again into two parts, one of them was used to carry out the comet assay and the other for the structural chromosome aberration test (Figure 1). The same experimental design was simultaneously implemented for control (untreated cells) and 1Gy-treated groups.

Insert Figure 1

Additional sets of cultures were designed using the traditional model. In this case, cells were exposed to a high challenging dose after pretreatment with only one adaptive dose exposure. Cells were irradiated with 10mGy while quiescent and then, with 1Gy of gamma rays during the 2^{nd} cycle. In this case, only structural chromosome aberration test was performed.

Cells used for both models were irradiated with nominal gamma ray doses of 10mGy and 1Gy with a high dose rate Microselectron Nucleotron® equipe, with a small ^{192}Ir source programmed by Indy® software, Silicon Graphics® computer. In order to obtain the programmed isodose curve, T-25 culture flasks were placed inside a polypropilene support suspended on an attenuating water layer within an acrylic chamber; this acrylic chamber was

placed on an acrylic plate with ten parallel needles separated each 10mm through which the ^{192}Ir source circulated.

The irradiation dose employed was 10mGy, take into account the dosimetry reported for previous investigations in our laboratory (Feinendegen 1999, Güerci *et al.,* 2004) and epidemiological studies (Barquinero *et al*. 1993, Paz-y-Miño *et al*. 1995, Balakrishnan and Rao 1999, Heimers 2000, Cardoso *et al*., 2001, Cavallo *et al*., 2002).

Comet assay

The comet assay was performed according to the method of Singh *et al* (1988) with some modifications (Tice and Strauss, 1995). Briefly, conventional slides were covered with a first 180 µl layer of 0.5% normal agarose (GIBCO-BRL, Los Angeles, USA). Then, a mix of 75 µl 0.5% low melting point agarose (GIBCO-BRL, Los Angeles, USA) and 15 µl cell suspension with approximately 15,000 cells was layered onto the slides, which were immediately covered with coverslips. After agarose solidification at 4º C for 5 min, coverslides were removed and slides were immersed overnight at 4º C in fresh lysing solution [(2.5 M NaCl - JT Baker, Phillipsburg, NJ, USA), 100 mM sodium ethylene diamine tetracetic (Na_2EDTA - JT Baker, Phillipsburg, NJ, USA), 10 mM hydroxymethil aminomethane tris (Tris, pH 10 - JT Baker, Phillipsburg, NJ, USA) containing 1% 4-octylphenol polyethoxylate (Triton X-100 - Sigma, St Louis, MO, USA) and 10% dimethylsulfoxide (Merck Química Argentina SAIC) added just before use]. Two slides from each group were prepared under dim light conditions. After lysis, slides were placed on a horizontal gel electrophoresis unit with fresh electrophoretic buffer (300 mM NaOH - Farmitalia Carlo Erba SpA, Milano, Italy, 1mM Na_2EDTA, pH > 13), left for DNA unwinding during 20 min, and then electrophoresed for 30 min at 1.25 V/cm (300 mA). This procedure was carried out at 4º C under dim light. After electrophoresis, slides were neutralized by washing three times with buffer (0.4M Tris, pH 7.5) every 5 min and then with distilled water. Slides were stained with SYBR Green I (Molecular Probes, Eugene, Oregon, USA) at recommended dilution (Ward and Marples, 2000).

A fluorescent microscope (Olympus BX40, with a 515-560nm excitation filter) connected to a Sony 3 CCD-IRIS color video camera was used for image observation at 400X magnification. Immediately after opening the microscope shutter to the computer monitor, each cell was photographed using the Image Pro-Plus 3.0 Program (Media Cybernetics, Silver Spring, MD, USA).

Based on the degree of DNA breakage, cells were classified according to their tail

length into five categories, ranging from 0 (no visible tail) to 4 (detectable head of the comet but most of the DNA in the tail). A sixth group including apoptotic cells (without detectable head) was considered (Olive 1996, Olive *et al*., 1998).

Radiation effect on the frequency of damaged cells was analyzed using the χ^2- test. Cells without damage (0 degree) were compared with those with low damage (1-2 degrees) and high damage (3-4 degrees and apoptosis).

Three separate experiments were performed for each experimental condition. A total of 300 images (100 per repetition) were scored per treatment.

Structural Chromosome aberration test

This test was used to analyze CA frequencies at the first metaphase after radiation. The lapse between radiation and fixation was 15-16 h. Colchicine (Sigma, St Louis, MO, USA) (0.1 µg/ml final concentration) was added to all cultures 2 h before fixation. Air dried slides were prepared following routine protocols.

Statistical analysis was performed using the χ^2 test.

All experiments were run twice in independent trials in order to assess reproducibility. A total of 400 metaphases per treatment were scored in coded slides.

Results

Comet assay

Table I shows the percentage of undamaged cells and those exhibiting genotoxic damage during the adapting serial radiation. The frequency of cells with low damage was significantly increased after 10mGy gamma-rays chronic treatment ($p<0.001$). No significant increase in the frequency of cells with severe damage and apoptosis was observed.

Insert Table I

Significant increase of cells with low and severe damage plus apoptosis and necrosis was found when comparing 1Gy treatment with controls ($p<0.001$). The same results were found for the combined treatment (chronic + high dose) when compared with 1Gy treated group (high dose) ($p<0.001$) (Table II) (Figure 2).

Insert Table II, Figure 2

Structural Chromosome aberration test

As expected (Güerci *et al.,* 2003), the 4-cycle ionizing radiation with 10mGy induced a significant increase in the frequency of abnormal metaphases when achromatic lesions were

scored ($p < 0.01$) in relation to controls (untreated). In the same way, 1Gy treatment significantly increased the appearance of abnormal metaphases ($p < 0.001$) in relation to controls. The frequency of abnormal metaphases for the combined treatment (chronic + high dose) was lower than for the respective controls (control + high dose). However, no significant decrease was found. When the different types of aberrations were considered, postreatment with 1Gy radiation decreased the frequency of dicentric chromosomes and chromosome rings (Table III).

Insert Table III

On the other hand, for the traditional model (only one low adapting dose), the frequency of abnormal metaphases in combined treatments (chronic + high dose) was similar to the one induced by only one high dose (1Gy) (Figure 3).

Insert Figure 3

Discussion

Exposure to low doses of radiation can prime an organism to withstand the stress of a subsequent exposure to higher doses of the same agent. This phenomenon has been called radioadaptive response (Venkat *et al.*, 2001). Several cytogenetic studies have been performed *in vitro* in order to analyze the adaptive response to ionizing radiation. However, most of the experiments were carried out with only one adapting dose (Sasaki *et at.,* 2002). We have just started experiments to simulate chronic exposure in order to induce this phenomenon *in vitro.* This approach will be applied to evaluate cytomolecular and cytogenetic DNA damage as a result of this response.

Our research studies have shown that low X-ray doses induce DNA damage in CHO-K1 cells previously exposed to the same dose (Güerci *et al*., 2003, 2004). Comet assay results confirmed this damage and showed that chronically induced DNA damage was higher than expected (Table I). Certain mechanisms reported such as the bystander effect (Mothersill and Seymour, 2003, 2004, Streffer 2004) could explain these results. Since the comet assay analysis is performed immediately after radiation, the damage degree observed for the combined treatment (chronic + high dose) was higher than that for the respective control (only acute exposure). The chromosomal aberrations test showed a decreased damage trend for our model (induced adaptive response) but not for the traditional one. These results could indicate that these effects take place after the induction of efficient DNA repair mechanisms leading to less residual damage and not after the induction of protective factors (enzymatic and non

enzymatic) that reduce initial DNA damage.

The radioadaptive response provides significant information for the risk assessment of the low dose and low dose rate exposures to ionizing radiation. Cells previously exposed to low dose radiation become resistant to mutations induction, CA and death, and are also more sensitive to malignant transformation (Sasaki, 1996).

On the other hand, induction of the adaptive response depends on a number of variables such as priming dose, time between adaptive and challenge exposures, radiation type, cell type and cell proliferation rate (Ikushima, 1989, United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR), 1996, Streffer, 2004 a.). In this sense, further research should be developed using not only different time periods between the adapting exposure to low gamma-ray doses and the acute dose, but also repair-deficient cell lines and repair inhibitors. The assessment of the repair mechanisms involved in the radioadaptive response is essential. In this sense, Ohnishi *et al* (2002) have reported that DNA-dependent protein-kinase activity might play an important role in the radioadaptive response, and Takahashi *et al* (2001) have observed that this mechanism might be due to the suppression of p53-mediated apoptosis.

Our findings contribute to explain the radioadaptive response as part of the complex interactive process of cell recover after low dose exposure to ionizing radiation. However, the molecular mechanism remains to be clarified (Sasaki *et al.,* 2002, Miyamoto *et al*., 2006).

Under these experimental conditions, our results show evidence about the protective effect of the chronic exposure to low gamma-ray doses against later high dose. However further studies are necessary to confirm this assumption.

Acknowledgements

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Figure 1. Schematic representation of the experimental description. CHO-K1 cells treated with 10 mGy per cycle of irradiation during the adapting serial of treatment and posttreatment with 1 Gy. Comet assay and structural chromosome aberration test were employed.

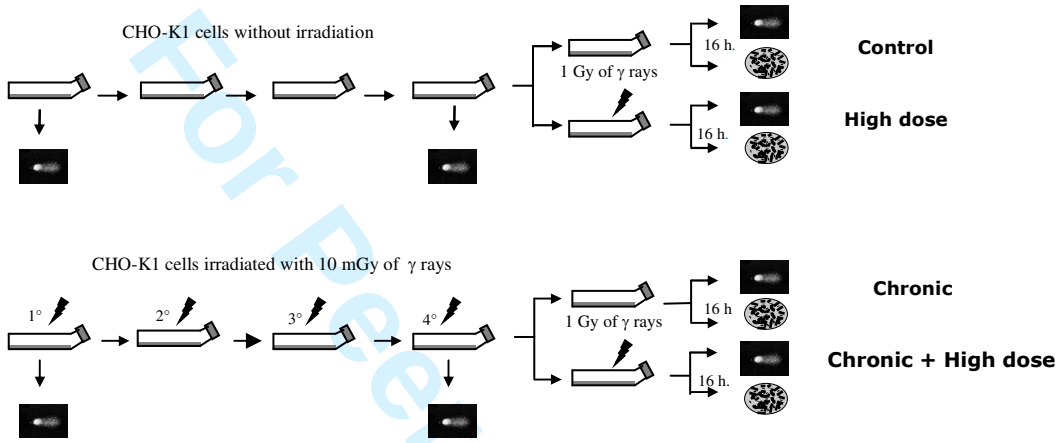

Figure 2. DNA damage in CHO-K1 cells treated with 10 mGy during 4 cycles of irradiation and posttreated with 1 Gy. For each experimental condition three separate experiments were performed. A total of 300 images per treatment were scored.

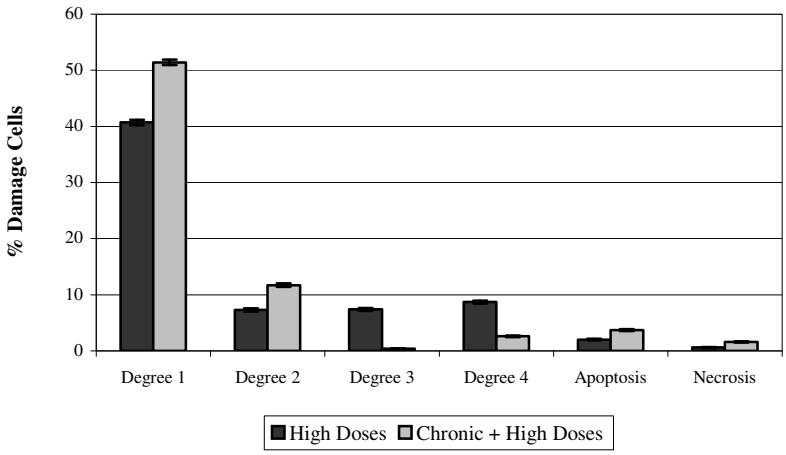

Figure 3. Frequency of abnormal metaphases in traditional model (one low adapting dose) (**A**) and non traditional model (4 cycles of irradiation with adapting dose) (**B**). For each experimental condition two separate experiments were performed. A total of 400 metaphases per treatment were scored. Black bars, with achromatic lesions. White bars, without achromatic lesions

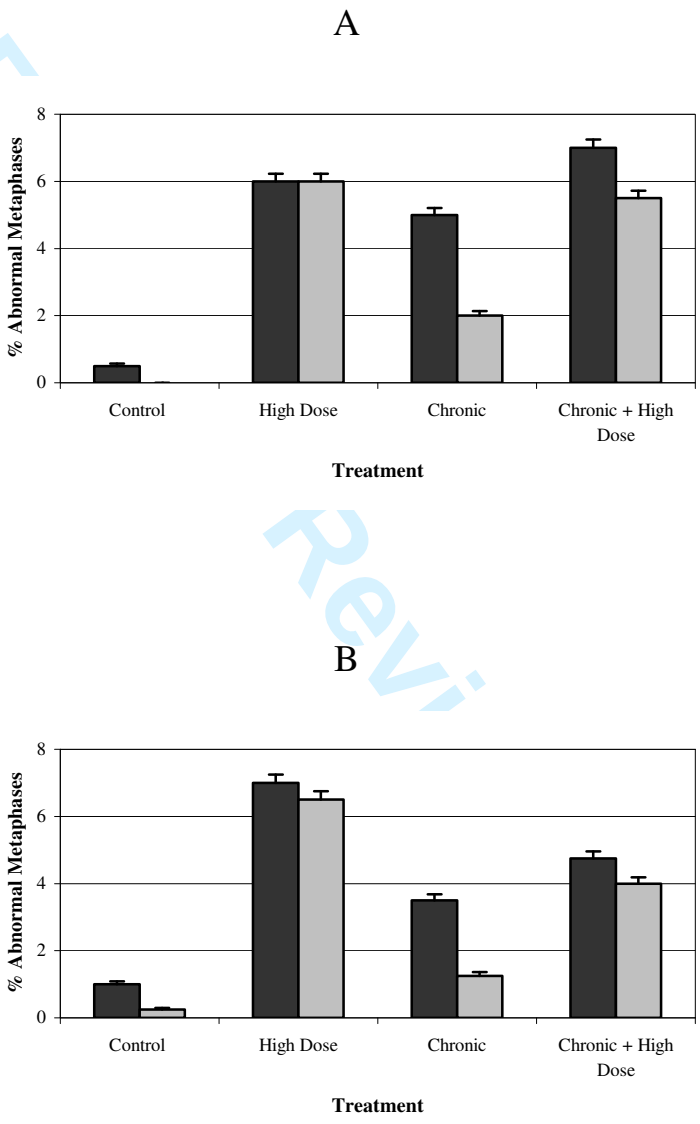

Table I. DNA damage in CHO-K1 cells treated with 10 mGy during the adapting serial radiation.

Radiation Order		Low DNA damage degree		Severe DNA damage degree		
	Degree 0	Degree 1	Degree 2	Degree 3	Degree 4	Apoptosis
Control - 1st	255	41	4	---	---	---
10 mGy - 1st	195	67	38	---	---	---
Control - 4 th	252	45	2	---	1	---
10 mGy - 4 th	81	190	27	---	---	2

Three separate experiments were performed for each experimental condition. A total of 300 images per treatment were scored.

Table II. DNA damage in CHO-K1 cells treated with 10 mGy during 4-cycle radiation and postreated with 1 Gy.

Treatment	Low DNA damage degree			Severe DNA damage degree			
	Degree 0	Degree 1	Degree 2	Degree 3	Degree 4	Apoptosis	Necrosis
Control	221	73	6	---	---	---	---
High Dose	100	122	22	22	26	6	2
Chronic	113	146	41	---	---	---	---
Chronic + High Dose	86	154	35	1	8	11	5

Three separate experiments were performed for each experimental condition. A total of 300 images per treatment were scored

Table III. Frequencies of structural chromosome aberrations in CHO-K1 cells treated with 10 mGy during 4-cycle of radiation and postreated with 1 Gy.

Treatment	Abnormal metaphases % Without gaps	Abnormal metaphases % With gaps	Chromosomal aberrations / 100 cells						
			AL [1]	B' [2]	B" [3]	RB' [4]	Frag [5]	DIC [6]	RING [7]
Control	0.25	1.0	1.0 (0.05)	---	---	---	---	0.25 (0.05)	---
High Dose	6.5	7.0	0.50 (0.05)	1.25 (0.11)	---	0.25 (0.05)	2.25 (0.15)	3.75 (0.19)	0.75 (0.08)
Chronic	1.25	3.5	2.25 (0.15)	0.25 (0.05)	0.25 (0.05)	---	0.75 (0.08)	0.25 (0.05)	---
Chronic + High Dose	4	4.75	0.75 (0.08)	1.25 (0.11)	0.75 (0.08)	---	2.75 (0.16)	2.25 (0.15)	---

Two separate experiments were performed for each experimental condition. A total of 400 metaphases per treatment were scored. Mean standard error is indicated between brackets.

1 AL: Achromatic lesions (gaps).
2 B': Chromatid breaks.
3 B": Isochromatid breaks.
4 RB': Chromatid exchanges.
5 Frag: Chromosome fragments.
6 DIC: Dicentric chromosomes.
7 RING: Chromosome rings.