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Status report of a systematic investigation on low-dose ionizing radiation effects in mammalian cells

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Summary. — In the last 15 years a growing interest in the biological effects induced by low doses of ionizing radiation has arisen in the scientific community, due to an increasing number of experimental evidences showing a plethora of non-linear effects occurring after low-dose irradiations. In particular, hyper-radiosensitivity and induced radioresistance (HRS/IRR) have been reported after exposure to low- and high-LET radiation, in human (normal and tumoural) and other mammalian cells *in vitro*. In this framework, Chinese hamster V79 cells, human primary fibroblasts (HFFF2) and murine embryonic fibroblasts (MEFs) were irradiated with broad-beams of protons in the dose range 0.1–5.0 Gy and at 1 Gy/min dose-rate. Cellular response has been evaluated in terms of cell survival, micronuclei induction, chromosomal aberrations and telomere length alterations. For comparison purpose, the same end-points were studied after X/γ-rays irradiation.

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PACS 87.53.Ay – Biophysical mechanisms of interaction.
PACS 87.53.Bn – Dosimetry/exposure assessment.
PACS 87.18.Gh – Cell-cell communication; collective behavior of motile cells.

1. – Introduction

In the last 15 years a continuously growing interest in the biological effects induced by low doses of ionizing radiation, of relevance to environmental and occupational radiation exposure, has arisen in the scientific community.

This is because recent experimental evidences *in vitro* have shown the presence of a plethora of phenomena occurring after low-dose irradiation (including hypersensitivity

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and induced radioresistance, adaptive response, and non-targeted phenomena like bystander effect and genomic instability), which might imply a non-linear behaviour of cancer risk curves in the low-dose region and question the validity of the Linear No-Threshold (LNT) model for cancer risk assessment through extrapolation from existing high-dose data [1-5].

In particular the evidence of "non-targeted" phenomena, implying that effects of ionizing radiation arise also in cells that are not themselves directly irradiated, contrasts the basic paradigm of radiobiology which considers the cell nuclear DNA as the target of ionizing radiation and the interaction of radiation with DNA as a primary requirement for cellular DNA damage induction. These effects are observed either in the descendants of irradiated cells (radiation induced genomic instability) or in cells that receive signals from irradiated cells of the same population (radiation-induced bystander effect).

Moreover recent experimental evidences following irradiation of human (normal and tumoural) and other mammalian cells *in vitro* show a deviation in the low-dose region of the cell survival curve behaviour from the standard linear or linear-quadratic response. This behaviour has been interpreted as a two-phase phenomenon whereby cells exhibit increased sensitivity at very low doses, named hypersensitivity (HRS) and then a relatively radioresistant phase termed increased radioresistance (IRR) as the dose increases. These data suggest the existence of cellular radioprotective mechanisms that may influence the shape of the survival response to single doses: very low doses seem to be more effective than larger doses above a threshold dose that triggers the radioprotective mechanism [6-8].

Similarly to IRR, it has been reported also an adaptive response to low-dose radiation which indicates that the cellular sensitivity to a dose of radiation decreases if the cells are exposed to a small radiation dose (5–40 cGy), the so-called priming or conditioning dose, a few hours prior to irradiation with a dose of few Gy (challenging dose). The priming dose activates cellular radioprotective mechanisms that might be in some way analogous to mechanisms promoting the induced radioresistance. The similarities between HRS/IRR after single doses and the adaptive response after a priming dose are many but the underlying mechanisms that control these effects are not fully characterized.

In this framework, the multi-year SHEILA (*Single Hit Effects Induced by Low-dose irrAdiation*) experiment, funded by INFN-CNS5, has focused its activity on the investigation of biological effects induced in human and rodent cells by accelerated ion beams of different quality, in terms of different biological end-points, including cell survival, chromosomal aberrations, micronuclei induction and alterations of telomere length, as a function of dose with particular emphasis on the low-dose region. Moreover, recently, to further investigate on the low-dose radiation response mechanisms it has been planned to analyse large-scale gene expression by DNA micro-array for identifying the complex multiple changes resulting from the interplay of radiation intra-cellular signalling pathways and to measure the ionic current flows by Patch-clamp technique for studying their role in the inter-cellular signalling pathways.

Different experimental irradiation approaches have been developed and are in use for mimicking *in vitro* the low-dose exposure and investigating the above-described phenomena, in terms of different biological end-points.

Briefly, they can be grouped in three major categories:

1) the conventional irradiation of cell populations with low doses of ionizing radiation (X/γ-rays, light and heavy ions) by using accelerated ion broad-beams at the INFN-LNL Radiobiology facilities [9, 10];

2) the conventional irradiation of cells but where a part of the population is shielded from exposure;

3) the use of charged-particles microbeams at the INFN-LNL Radiobiology facilities which make it possible to irradiate cell individually with a predetermined number of charged particles [11].

In this paper preliminary results related to irradiations of rodent and human cells (V79, MEFs, HFFF2) with broad-beams of protons of different energy performed at INFN - Laboratori Nazionali di Legnaro are presented. X-/γ-rays irradiation has been also performed for comparison purpose.

In particular, preliminary results on cell survival, chromosomal aberrations, micronuclei induction and telomere length alterations, as a function of dose and radiation quality, are reported and briefly discussed.

Chromosomal aberrations and micronuclei measurements were performed as well-established biomarkers of DNA damage, which turn useful to be correlated with cell survival. The analysis of the telomere length was considered as reporter of the sensitivity of chromosome components to radiations. It has been reported that radiation-induced DNA lesions may promote the loss of functional telomeres and, consequently, chromosome fusions and chromosome instability. Telomeres, which consist of tandem TTAGGG repeats and associated proteins play an essential function, that is chromosome capping. In fact, telomeres protect chromosomes ends from degradation and fusion events and may take part in the healing of chromosome or chromatid breaks produced by DNA damage. Telomere shortening can trigger cellular growth arrest.

Furthermore it was evaluated whether telomere may also represent a useful biomarker of exposure to radiations of different quality, particularly at the low-dose level of exposure.

2. – Materials and methods

2˙1. *Cells and culture conditions.* – Chinese hamster embryonic lung fibroblasts (V79 cells; ECACC, UK), human primary foetal foreskin fibroblasts (HFFF2 cells; ECACC, UK) and Murine embryonic fibroblasts (MEFs) were used.

V79 cells were grown in monolayer in D-MEM medium supplemented with 10% heat-inactivated foetal bovine serum, antibiotics and L-glutamine.

HFFF2 cells were cultured in D-MEM medium supplemented with 10% of either foetal calf serum or foetal bovine serum, antibiotics and L-glutamine.

MEFs cells were grown in high glucose D-MEM medium supplemented with 20% foetal bovine serum, antibiotics and L-glutamine.

All the cell cultures were incubated in a humidified incubator at 37 °C in an atmosphere of 5% CO_2 in air.

2˙2. *Irradiation.* – 12–18 hours (depending on the cell line) before irradiation cells were plated as a monolayer on T25 flasks for γ-irradiation and on specially designed stainless-steel Petri dishes [9] for proton irradiation.

Chinese hamster V79 cells were irradiated with Co-60 γ-rays and broad-beams of protons of different energies in the dose range 0.1–5.0 Gy and at 1 Gy/min dose-rate.

HFFF2 and MEFs cells were irradiated with protons and X-rays in the dose range of 0.25–4 Gy, at 1 Gy/min and 70 cGy/min dose-rate, respectively. Proton irradiations were performed at the Radiobiology facility of the INFN-LNL 7 MV Van de Graaff CN

accelerator [9]. Proton energies of 0.8 MeV and 5 MeV, at the cell entrance surface (corresponding to LET values, in muscle tissue, of 28.5 keV/μm and 7.7 keV/μm, respectively) have been used. All proton irradiation experiments were performed in the so-called track-segment conditions. Irradiation facility, beam dosimetry and irradiation modalities have been described in detail elsewhere [9, 12].

Gamma irradiations have been performed at the Co-60 γ-beam facility of the CNR-FRAE Institute at INFN-LNL. Experiments with X-rays were carried out by using a 250 kV, 6 mA apparatus equipped with a 0.2 mm copper filter (Gilardoni, Italy) as described elsewhere [12, 13].

Sham irradiated cells were used in all the experiments as control (unirradiated) cells.

2˙3. *Cell survival.* – Cell survival has been tested by colony-forming assay: after irradiation, for each dose point, including unirradiated control cells, V79 cells were washed with PBS buffer, trypsinized and counted, diluted with fresh medium and replated at appropriate concentration in 6-cm Petri dishes to determine the surviving fraction. After 7 days of growth at 37 °C, the cells were fixed and stained, and visible colonies with more than 50 cells were counted as survivors.

2˙4. *Cytogenetic analysis*
Micronuclei induction

After irradiation, for each dose point considered, including unirradiated control cells, V79 and HFFF2 cells were trypsinized, counted, diluted with fresh medium and replated. Eight hours later, fresh medium containing 3 μg/ml Cytochalasin-B (Sigma) was added. Twenty-four hours later cells were washed once with PBS, detached by trypsinization and cytospunned.

Then HFFF2 cells were fixed 30 min in absolute methanol at −20 °C, air dried, stained with 0.2 μg/ml DAPI and mounted with anti-fade solution (Vector); V79 cells were fixed with methanol-acetic acid solution and stained with 10 μg/ml acridine orange for 2 min. The induction of MN was evaluated in at least 500 cells per experimental point (including control cells) with a fluorescent microscope (under a blue-violet illumination).

Chromosomal aberrations

For chromosomal aberrations analysis, post-irradiated V79 cells and unirradiated control cells, were incubated in Colcemid-containing medium (0.025 μg/ml) for 2 hours to arrest cells in the mitotic phase of the cell cycle. Then cells were washed, collected by trypsinization and added with 75 mM KCl, fixed with methanol-acetic acid solution and stained with 3% Giemsa. For chromosomal aberration yield evaluation, 100 metaphases were scored per each dose (including control cells).

2˙5. *Q-FISH in interphase nuclei.* – To evaluate radiation-induced alterations of telomere length, Q-FISH staining was performed on interphase nuclei of HFFF2 and MEFs with fluorescent PNA telomeric probe and telomere size was analysed with TFL-TELO software (kindly provided by Peter Lansdorp, British Columbia Cancer Center, Vancouver, Canada). The fluorescence intensity of telomeres was expressed in arbitrary units (a.u.f).

3. – Results and discussion

3˙1. *Clonogenic death, chromosomal aberration and micronuclei induction in V79 cell.* – The gathered cell surviving fractions are reported as a function of dose in figs. 1 and 2.

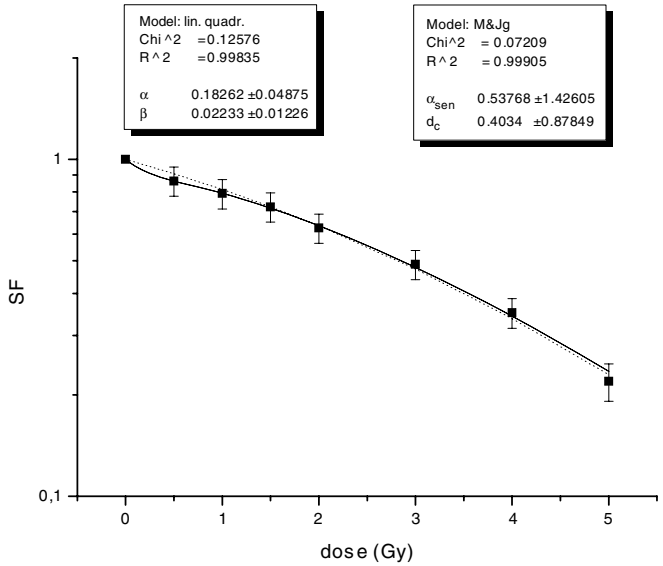

Fig. 1. – V79 cell survival after Co-60 gamma-rays irradiation. Each data point is the mean of 9 independent experiments. Error bars represent the standard error (see text).

Each data point is the mean of 3–9 independent experiments. Error bars represent the standard error (or 10% of the mean if the standard error is minor than 10%).

Cell survival data after Co-60 γ-rays irradiations (see fig. 1) show a deviation in the low-dose region (below 0.5 Gy) of the survival curve from the conventional behaviour

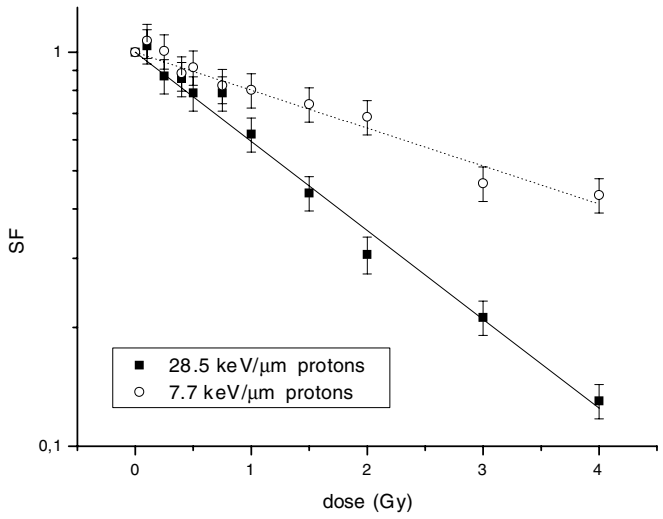

Fig. 2. – V79 cell survival after 7.7 and 28.5 keV/μm proton irradiation. Each data point is the mean of 3 (7.7 keV/μm, protons) or 7 (28.5 keV/μm, protons) independent experiments. Error bars represent the standard error (see text).

back-extrapolated from high-dose data using the linear-quadratic model and are in agreement with the results obtained in similar studies [6, 14]. Such studies suggested that the linear-quadratic (LQ, $S = S_0 \exp[-\alpha D - \beta D^2]$) relationship does not describe correctly the cell survival curve in the low-dose region (below 1 Gy). A modified LQ equation has been proposed, in which α varies with dose, to model this response [15]. According to this hypothesis, the γ-rays survival in the higher dose region (1–5 Gy) was assumed to follow the linear quadratic relationship from which estimates of α and β can be obtained and then used (α becoming α_{res}) to fit the experimental data in the lower dose region with the "Induced Repair (IR) model":

$$SF = \exp\left[-\alpha_{\mathrm{res}}\left[1 + \left(\frac{\alpha_{\mathrm{s}}}{\alpha_{\mathrm{res}}} - 1\right) e^{-d/d_c}\right] d - \beta d^2\right], \tag{1}$$

where d is dose, α_{s} represents the low-dose value of α, α_{res} is the value extrapolated from the conventional high-dose response, d_{c} is the "transition" (or threshold) dose at which the change from the very low-dose HRS to the IRR response occurs, and β is a constant as in the LQ equation.

By using the IR model to fit γ-rays survival data here reported, the curve slope in the hypersensitivity region is $\alpha_{\mathrm{s}}^{\gamma\mathrm{R}} = 0.5\,\mathrm{Gy}^{-1}$ ($\alpha_{\mathrm{s}}^{\gamma\mathrm{R}}/\alpha_{\mathrm{res}} = 3$). Moreover the triggering of radioresistance for γ-rays and therefore the slope reversal from HRS to IRR region occurs at a threshold dose that is $d_{\mathrm{c}} = 0.4\,\mathrm{Gy}$, in agreement with data in the literature [6, 8, 14, 16]. For doses greater than 1 Gy, survival decreases logarithmically with dose following a linear-quadratic curve ($\alpha_{\gamma\mathrm{R}} = (0.18 \pm 0.05)\,\mathrm{Gy}^{-1}$; $\beta_{\gamma\mathrm{R}} = (0.02 \pm 0.01)\,\mathrm{Gy}^{-2}$).

After 7.7 and 28.5 keV/μm proton irradiations, survival fraction decreases linearly with dose and data were fitted with the linear model ($S = S_0 \exp[-\alpha D]$; $\alpha_{\mathrm{p}} = (0.22 \pm 0.02)\,\mathrm{Gy}^{-1}$ and $\alpha_{\mathrm{p}} = (0.53 \pm 0.02)\,\mathrm{Gy}^{-1}$, respectively). No clear evidence of an HRS/IRR substructure can be identified in the low-dose region (see fig. 2).

The HRS/IRR phenomenon, with a deviation in the survival curve behaviour in the low-dose region, has been demonstrated to date in over 40 cell lines (tumour, normal and established cell lines) after low-LET irradiation (X/γ-rays, plateau pions) with the "transition" dose in the range 0.20–0.60 Gy, depending on the cell line.

Moreover the biphasic HRS/IRR behaviour has been found for survival curves of V79 cells after high-LET irradiations with alpha-particles of different energies, the deviation being more evident with increasing LET [17], and carbon ions [18]. On the other hand, no deviation has been reported after irradiation with other high-LET radiation, like neutron [6] and peak pions [19]. In the same way no evidence of substructure and LQ behaviour were measured after 70 MeV proton irradiation [20].

In this literature background, the data here presented add further information towards a possible correlation between HRS/IRR phenomena and radiation quality (type and energy/LET). Proton beams used in this study turn as a useful tool for such an aim: in fact 7.7 keV/μm protons are low-LET charged particles, with biological effectiveness close to γ-rays, while 28.5 keV/μm protons are high-LET charged particles corresponding to the peak value of proton RBE-LET curve [21, 22].

In order to elucidate which mechanisms are underlying HRS/IRR phenomena after low-dose exposures, cellular response has been also studied in terms of chromosomal aberrations and micronuclei induced by both γ-rays and 28.5 keV/μm proton irradiations as a function of dose. Data gathered up to now are related to 1-3 experiments and therefore are very preliminary. The yields of aberrations per cell and micronuclei per binucleated cell increase linearly with dose after both gamma and proton irradiations

TABLE I. – *Unstable chromosomal aberrations by Giemsa staining analysis in V79 cells irradiated with ^{60}Co γ-rays and 28.5 keV/μm protons. Each data point represents three independent experiments for γ-rays, a single experiment for protons.*

		Aberration types (per 100 cells)			
	Dose (Gy)	Acentric fragments	Dicentrics	Rings	Total
^{60}Co γ-rays	0	4.7 ± 0.9	0	0	4.7 ± 0.9
	0.5	14 ± 4	2.0 ± 0.6	1 ± 1	17 ± 5
	0.75	7 ± 1	4.1 ± 0.6	1.0 ± 0.6	12 ± 1
	1	9 ± 1	5.1 ± 0.6	3.9 ± 0.7	18 ± 2
	1.5	15.1 ± 0.7	6.5 ± 0.5	2.3 ± 0.9	23.9 ± 0.7
	2	14 ± 2	10.2 ± 0.8	5.3 ± 0.7	30 ± 2
	3	18 ± 4	13 ± 1	11 ± 1	42 ± 4
	4	26.4 ± 0.7	17 ± 1	7.8 ± 0.6	51 ± 3
	5	44 ± 3	18.7 ± 0.7	11.3 ± 0.7	74 ± 2
	6	42 ± 1	33 ± 1	8.9 ± 0.6	84 ± 1
28.5 keV/μm protons	0	11 ± 1	0	0	11 ± 1
	0.1	13 ± 1	8 ± 0	0	21 ± 1
	0.25	21 ± 5	7 ± 1	1 ± 1	29 ± 5
	0.4	27 ± 11	10 ± 6	5 ± 1	42 ± 4
	0.5	22 ± 2	11 ± 1	6.5 ± 0.5	39 ± 3
	0.75	27 ± 11	15 ± 1	3 ± 1	45 ± 11
	1	46 ± 0	16 ± 2	5.5 ± 0.5	67 ± 2
	2	69 ± 13	34 ± 0	12 ± 2	115 ± 15

(tables I and II). In particular, the chromosomal aberration yield increases more rapidly for proton than for γ-rays. Data related to gamma irradiation show a deviation from the linear dose-response curve of acentric fragments per cell (and therefore of total aberrations per cell) and micronuclei at the dose of 0.5 Gy, corresponding to the threshold dose where the shape reversal of cell survival curve occurs (tables I and II).

3˙2. *Micronuclei induction and telomere length alterations in HFFF2 and MEFs cells.* – The MN-assay has been used to measure DNA damage after exposure of HFFF2 cells to X-rays or protons in the dose range of 0.25–2 Gy. Preliminary results indicated that the frequency of MN induced by 28.5 keV/μm protons was far higher than in X-ray–treated cells (not shown). In agreement with the clastogenic effect of protons observed in Cl-1 Chinese hamster cells [12], we found a clear LET-dependence in the induction of MN in cytochalasin-arrested binucleated cells, with the effectiveness of 28.5 keV/μm protons at least two times greater than that observed in X-ray–treated samples. The MN assay discriminated between the two types of radiations at doses as low as 0.25 Gy (not shown). As reported here above, this difference is not so evident in MN-dose response curve measured for V79 cells, suggesting a cell-type dependence. Contrastingly to our preliminary results obtained scoring MN and chromosomal aberrations in the dose-region below 0.5 Gy for V79-irradiated cells, by means of MN assay we could not detect HRS neither for X-rays nor for protons in HFFF2 cells. This seems to indicate cell-type dependence in HRS response to radiations of different quality.

TABLE II. – *Frequency of micronuclei (Acridine orange staining) in V79 cells irradiated with* ^{60}Co *γ-rays and 28.5 keV/μm protons. Each data point represents three independent experiments for γ-rays, a single experiment for protons.*

		Number of micronuclei (MN) per binucleated (BN) cell (%)				
	Dose (Gy)	1	2	3	> 3	Total
^{60}Co γ-rays	0	0.027 ± 0.004	0	0	0	0.027 ± 0.004
	0.5	0.088 ± 0.003	0.013 ± 0.002	0	0	0.115 ± 0.006
	0.75	0.09 ± 0.02	0.005 ± 0	0	0	0.10 ± 0.02
	1	0.110 ± 0.008	0.005 ± 0	0	0	0.120 ± 0.008
	1.5	0.105 ± 0.007	0.027 ± 0.004	0.008 ± 0.002	0.005 ± 0.003	0.204 ± 0.020
	2	0.114 ± 0.003	0.032 ± 0.004	0.014 ± 0.002	0.011 ± 0.003	0.26 ± 0.03
	3	0.16 ± 0.02	0.065 ± 0.008	0.018 ± 0.003	0.013 ± 0.003	0.39 ± 0.04
	4	0.256 ± 0.007	0.12 ± 0.01	0.040 ± 0.006	0.047 ± 0.004	0.80 ± 0.01
	5	0.242 ± 0.002	0.13 ± 0.13	0.070 ± 0.003	0.098 ± 0.002	1.10 ± 0.02
	6	0.255 ± 0.009	0.163 ± 0.009	0.096 ± 0.006	0.112 ± 0.006	1.32 ± 0.01
28.5 keV/μm protons	0	0.013 ± 0.001	0	0	0	0.013 ± 0.001
	0.1	0.018 ± 0.006	0	0	0	0.018 ± 0.006
	0.25	0.033 ± 0.003	0.001 ± 0.001	0	0	0.035 ± 0.005
	0.4	0.035 ± 0.009	0.001 ± 0.001	0	0	0.04 ± 0.01
	0.5	0.036 ± 0.004	0.003 ± 0.001	0	0	0.042 ± 0.002
	0.75	0.054 ± 0.006	0.005 ± 0.001	0	0	0.064 ± 0.008
	1	0.07 ± 0.02	0.013 ± 0.001	0.002 ± 0	0.001 ± 0.001	0.10 ± 0.02
	2	0.123 ± 0.015	0.022 ± 0.002	0.007 ± 0.003	0	0.188 ± 0.002

Human primary fibroblasts and mouse embryo fibroblasts have been used to study whether exposure to X-rays or to low-energy protons alter the telomere length as evaluated by using the Q-FISH technique in MEFs (fig. 3) and in HFFF2 fibroblasts (fig. 4) interphase nuclei. The analysis has been performed at two different harvesting times in order to ascertain a possible delayed telomere dysfunction. Alterations in telomere length appeared as a biomarker of lower sensitivity than MN, needing higher doses of radiation to be modulated. The obtained results show a clear effect of radiation quality, in agreement with our previous data [23, 24]. The main difference was observed in cells collected shortly after irradiation (24 hours), where telomere elongation was detected only for proton irradiation in both types of cells used. The different response in telomere elongation as detected at 24 hours after irradiation could be related to the quality of radiation used and the "complexity" of DNA damage: a more complex damage could be responsible for an earlier activation of processes related to elongation. Taking into account our previous results, showing that neither X-rays nor protons were able to activate telomerase [25], telomere length modulation could be due to an alternative mechanism of telomere lengthening based on recombination between telomere sequences [26]. To test such a hypothesis, a set of experiments have been planned for assessing recombination process by means of Chromosome Orientation FISH technique (CO-FISH) [27]. It could be speculated that recombination between sister chromatids might be activated in a different way by low- and high-LET as a result of DNA damage severity. Contrastingly to that observed at 24 hours, 15 days after treatment, an increased length of telomeres

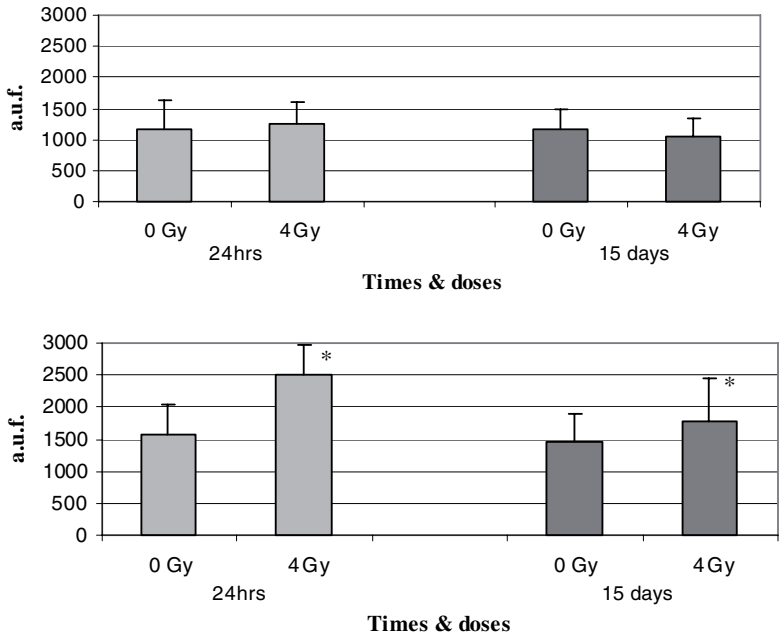

Fig. 3. – Telomere length in MEFs interphase nuclei expressed as arbitrary unit of fluorescence (a.u.f.). after X-irradiation (upper panel) and proton irradiation (lower panel) ($^*p < 0.01$, Student's T-test).

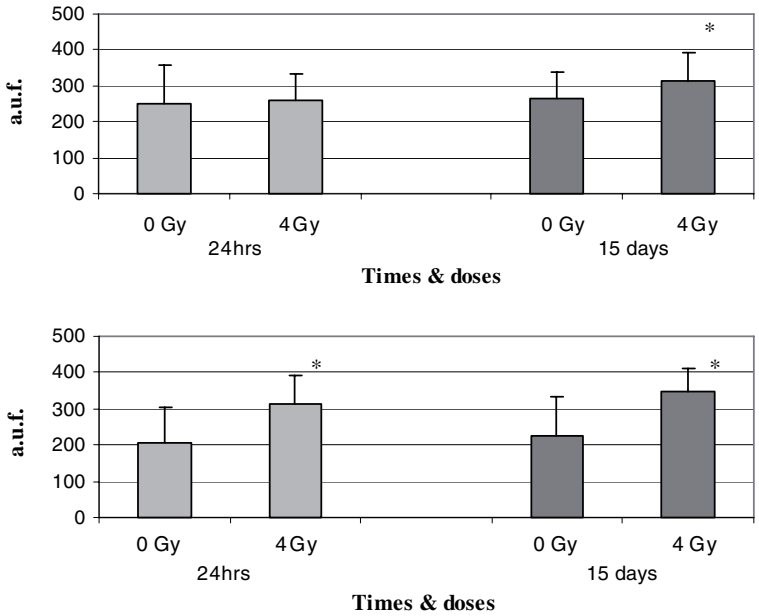

Fig. 4. – Telomere length in HFFF2 interphase nuclei expressed as arbitrary unit of fluorescence (a.u.f.). after X-irradiation (upper panel) and proton irradiation (lower panel) ($^*p < 0.01$, Student's T-test).

was detected for both types of radiation in HFFF2 fibroblasts, whereas in MEFs, such response was appreciated only for protons. The delayed X-ray–induced elongation may occur as result of a selective process for more radioresistant cells present in the cellular population at the time of irradiation, assuming a relationship between telomere length and radioresistance. To support this hypothesis, it remains to be investigated whether the mean of telomere length in HFFF2 irradiated-surviving cells is higher than the mean of non-irradiated cells. In this context, a detailed analysis of telomere length distribution in untreated MEFs cells may also help to shed light on the cell-type differences observed in human and mouse fibroblasts at 15 days from irradiation.

In summary, the preliminary results on cell survival, chromosomal aberrations and micronuclei induction in V79 cells here reported seem to show that HRS/IRR phenomenon occurs after low-dose irradiations with γ-rays but not either with high- or low-LET protons, indicating this phenomenon is strictly related to radiation quality. Furthermore, cell-type differences may also play a role in HRS/IRR, as shown by the different behaviour of V79 and HFFF2 with respect to MN induction for doses below 0.5 Gy.

Both the MN test and the analysis of telomeres were proven to be useful tools to discriminate between low- and high-LET radiations, though with different sensitivity in the low-dose range.

Further experiments are in progress to confirm and extend the reported findings.

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