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Comparative study of the radiobiological effects induced on adherent *vs* suspended cells by BNCT, neutrons and gamma rays treatments

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HIGHLIGHTS

- Cell survival and cell cycle distribution of suspended and adherent irradiated cells.
- Comparative irradiation set ups for *in vitro* BNCT studies
- Optimization of cell survival studies related to BNCT treatment.

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ABSTRACT

The present work is part of a preclinical *in vitro* study to assess the efficacy of BNCT applied to liver or lung coloncarcinoma metastases and to limb osteosarcoma.

Adherent growing cell lines can be irradiated as adherent to the culture flasks or as cell suspensions, differences in radio-sensitivity of the two modalities of radiation exposure have been investigated. Dose related cell survival and cell cycle perturbation results evidenced that the radiosensitivity of adherent cells is higher than that of the suspended ones.

1. Introduction

In vitro experiments can give important information about BNCT feasibility and dosimetry: currently, cell survival curves as a function of absorbed dose have been employed to calculate RBE and CBE factors. These factors are used to calculate "equivalent dose", in order to compare BNCT biological dose to the photon dose of conventional radiotherapy. Although it is recognized that this method of dose calculation produces artificially high values and that fixed RBE and CBE factors are not a suitable strategy to calculate mixed-field dose, survival curves are still a solid tool to prove BNCT ability to kill large populations of tumour cells. Recently, a new method to calculate *isoeffective dose* has been published (González and Santa Cruz, 2012): the idea is to equal the Tumour Control Probability (TCP) due to BNCT and to photons instead of fixing a single endpoint in the survival curves. Even if there is a debate about the capacity to represent the tumor response *in vivo*, dose dependant cell survival curves can be employed as an input of a more precise formalism for dose calculation. This represents an advance with respect to the fixed factors adopted presently. The purpose of this paper is to show the differences in the results obtained when cells are irradiated in different conditions, stressing that the comparison of survival curves obtained in different laboratories is not straightforward.

Clonogenic assay, established more than fifty years ago (Puck and Marcus, 1956), has become an accepted technique in radiation

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biology to determine the radiation sensitivity of different cell lines (Munshi et al., 2005; Franken et al., 2006). The methodology for plating assay of adherent growing cell lines provides that cells can be irradiated either before or after preparation of a suspension of single cells (Harding et al., 2013). Many papers aimed at evaluating the radiation effects on cell lines exposed to BNCT treatment by plating assay, report that irradiation has been performed on cell suspensions placed into teflon tubes (Gabel et al., 1984; Coderre et al., 1993; Tilly et al., 1996; Kinashi et al., 2002; Hsieh et al., 2005; Seki et al., 2015). Other studies with the same aim have been performed irradiating cells while adherent to the culture flasks or plates (Davis et al., 1970; Kamida et al., 2008; Phoenix et al., 2009). Despite considered as the most adequate method to test cell radiosensitivity, cloning assay is a material and time consuming method, therefore alternative assays have been checked (Wittig et al., 1998; Sieuwerts et al., 1995). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay, a well known test usually used to study chemo-sensitivity or toxicity, was compared to clonogenic assay resulting in a good correlation (Buch et al., 2012; Kawada et al., 2002). MTT test performed after irradiation of adherent cells was applied also to cell survival studies after BNCT treatment (Dagrosa et al., 2011). The use of different laboratory protocols for *in vitro* cell irradiation can have consequences in the calculation of the dose, making it difficult to compare results (Mackonis et al., 2012). In the frame of *in vitro* pre-clinical studies, aimed to evaluate the efficacy and applicability of BNCT to diffused hepatic and lung coloncarcinoma (Zonta et al., 2009; Bakeine et al., 2009) and to limb osteosarcoma (Ferrari et al., 2011), we have investigated the radio-sensitivity differences, in terms of cell survival and cell cycle perturbations, of cell lines exposed to Cobalt-60 γ-rays, neutrons and neutrons after BPA absorption, following two exposure modalities: adherent to the culture flask and as cell suspension inside vials. The main aim of this work is to assess whether the irradiation modality can affect cell survival and has therefore to be considered in the evaluation and comparison of inter/intra-laboratories results.

2. Materials and methods

2.1. Cell cultures

The rat colon adenocarcinoma DHD/K12/TRb (DHD) and the rat osteosarcoma UMR-106 (UMR) cell lines, were obtained from the European Collection of Cell Cultures (ECACC, UK). DHD cells were grown as monolayers in 75-cm^2 filter flasks at 37 °C in humidified 5% CO_2 air in a medium composed by HAM'S F10 and DMEM low glucose (1:1 v/v), while UMR cells, at the same conditions, in DMEM high glucose. Both media were supplemented with 10% Foetal Bovine Serum (FBS) and 40 μg/ml gentamicine (Euroclone, Italy). Cells were replated at subconfluency 48 h prior to any treatment. At the time of irradiation, the cell populations were non-confluent, asyncronous and continuously growing.

2.2. Experimental design

DHD and UMR cells were treated with ^{60}Cobalt γ-rays (^{60}Co), neutrons and BNCT following two different irradiation set ups: adherent to the culture flasks and detached as cell suspensions inside vials.

In case of BNCT, a solution of fructose-L-^{10}Boronophenilalanine (^{10}BPA) (Hummercup AB, Stockholm, Sweden) was used for intracellular boron enrichment.

The dose-response cell survival rates after irradiation of both cell lines were determined using the conventional clonogenic assay.

The radiation induced cell cycle perturbations were monitored on DHD cells by flow cytometric DNA analysis performed at fixed times post-irradiation.

2.3. Intracellular boron enrichment

Cells were cultured in medium enriched with the boronated carrier, at the concentration of 80 μg/ml for 4 h (Ferrari et al., 2009). Intracellular boron uptake was evaluated by neutron autoradiography on non irradiated cells, by seeding 5×10^6 cells, on Mylar® disks as elsewhere described (Gadan et al., 2012).

2.4. Irradiation set-ups and dose calculations

2.4.1. ^{60}Co irradiation

Adherent cells: cells were submitted to irradiation in 75-cm^2 culture flasks containing 20 ml of renewed culture medium.

Suspended cells: subconfluent cells were trypsinized, counted and transferred at concentration of 5×10^6/ml into 1 ml polythene tubes then housed in a special plexiglas stand.

Irradiation was performed in a ^{60}Co apparatus (field size: 32 cm × 32 cm; SSD: 78.5 cm; dose rate: 0.815 Gy/min) delivering doses of 3.5, 5, 7 and 10 Gy in electronic equilibrium conditions.

2.4.2. Neutron irradiation

Adherent cells: ^{10}BPA treated and untreated cells were submitted to irradiation in the culture flasks, after medium replacing as previously reported for ^{60}Co treatment.

Suspended cells: at the end of the fixed 4 h of incubation time the ^{10}BPA enriched medium was removed and after three PBS washings cells were processed as described for ^{60}Co irradiation and transferred into polythene tubes then housed in a Teflon stand to be exposed to the neutron flux. ^{10}BPA untreated samples were similarly processed. Cells were irradiated in the Thermal Column of the Triga Mark II reactor of the University of Pavia, for 10 min, at reactor powers from 1 to 250 kW.

UMR and DHD cells treated with 80 ppm of BPA for 4 hours before irradiation showed intracellular concentration in the range 15–40 ppm. UMR and DHD not treated with BPA, showed a background boron concentration between 1 and 3 ppm.

Doses delivered by neutron and BNCT treatments to suspended and adherent cells were calculated by Monte Carlo simulations (MCNP6 code). As the cell layer in the flasks and the cell pellet in the vials have very small volumes, the calculations were conducted in two steps. First of all the reaction rate of (n,p) reactions in nitrogen and (n,alpha) reactions in boron (for 1 ppm) was calculated with a simulation of the set-up inside the thermal column of the reactor. Cells were simulated as a layer of average adult soft tissue according to ICRU-44, with density 1.05 g/cm^3 and 2.3% (weigh percentage) of nitrogen (ICRU, 1989). Afterwards, protons, alpha particles and Li ions were generated and transported inside the cell volumes and the dose (from the actual deposited energy) was calculated. The normalization was then applied multiplying by boron concentration measured in each experiment and by the corresponding reaction rate. Gamma dose was calculated in the same modality, considering both the gamma radiation present in the irradiation facility (structural component) and the 2.2 MeV photons generated by neutron capture in H. These photons were generated inside the cells and the materials of the cell holders (culture medium, flasks and vials) and then transported also considering electrons. No assumption about charged particles equilibrium was necessary, and a dosimetry as precise as possible was obtained for each configuration.

2.5. Cell survival

After irradiation, *adherent cells* were suspended by trypsinization, counted, diluted in growth medium and plated into plastic 60-mm Petri dishes for clonogenic survival assay. Three different dilutions were used for each experimental condition and five Petri dishes per dilution were plated at cellular densities ranging from 50 to 5000 depending on the expected cell survival. Cells were allowed to grow for about seven to ten days until discrete colonies formation, replacing medium after five days. Dishes were then washed in Hank's phosphate buffer (Euroclone, Italy), fixed in 70% ethanol and then stained with toluidine blue for up to 50 cells colony counting. Surviving fractions were calculated by dividing the mean value of the plating efficiency of irradiated samples by those of non irradiated controls. Survival of cells was then plotted against the absorbed dose.

In the *cell suspension* irradiation set up, soon after treatment, cells were properly diluted in complete medium and seeded as for experiments on adherent cells.

2.6. DNA analysis

Cell cycle distribution was studied on DHD cells exposed to all the tested ^{60}Co doses and to neutrons only and BNCT at the highest reactor power for 10 min. Simultaneously to Petri plating for cell survival assay, DHD cells were also seeded in 75-cm^2 flasks for subsequent cell cycle DNA analysis. Cell cycle distribution was monitored for up to seven days post-irradiation by seeding two flasks for each subsequent observation day. Flasks intended for 24 h and 48 h analyses were reseeded with 1×10^6 cells while those for 5 and 7 days analyses respectively with 5×10^5 and 2.5×10^5. The concentration of cell seeding was experimentally

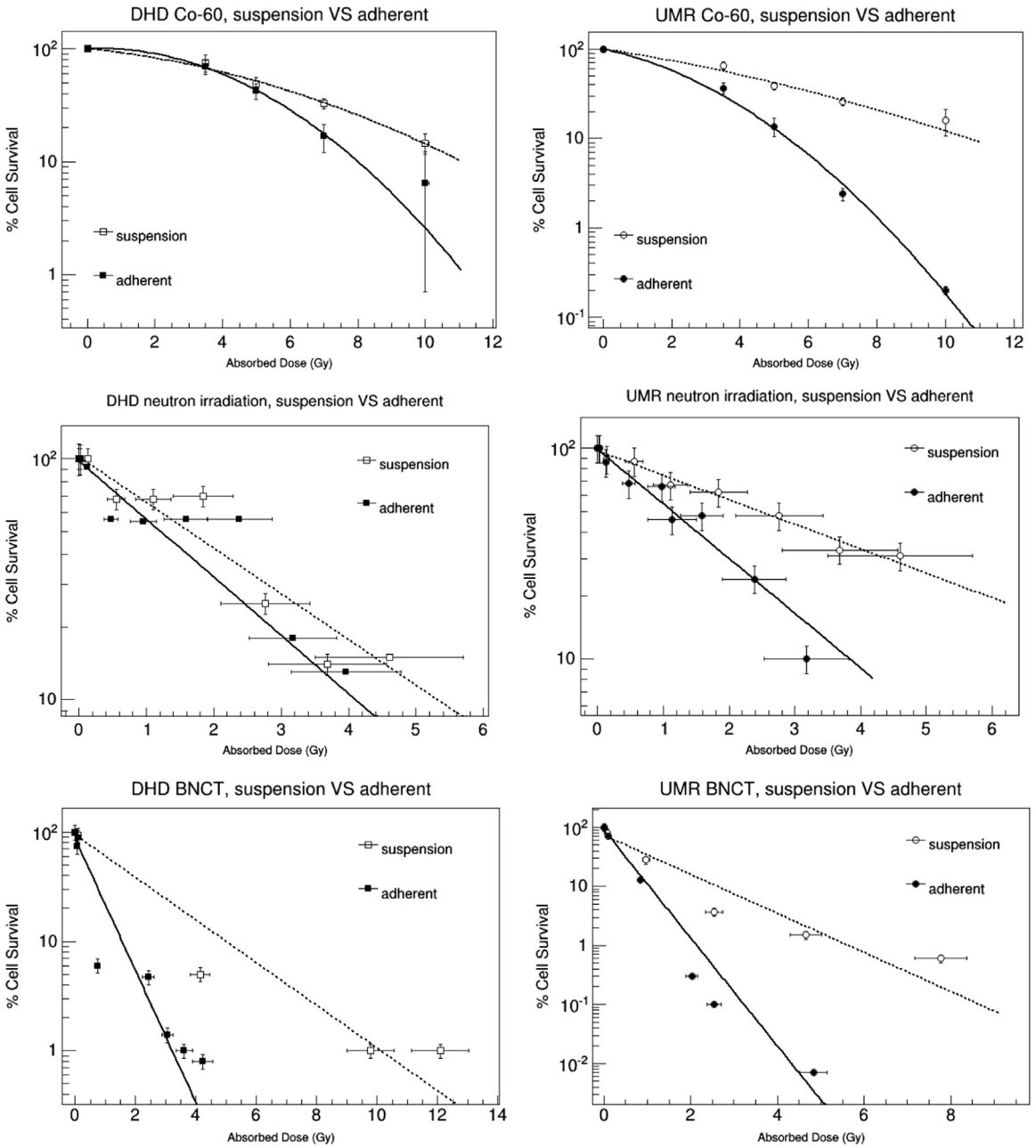

Fig. 1. Survival curves of suspended (o) and adherent (•) irradiated DHD cells (left) and of UMR cells (right) after exposure to ^{60}Co γ-rays (top), thermal neutrons only (middle) and to BNCT (below). Each point represents the mean ± 1 SD. The solid line fits survival of adherent cells while the dotted one that of suspended cells.

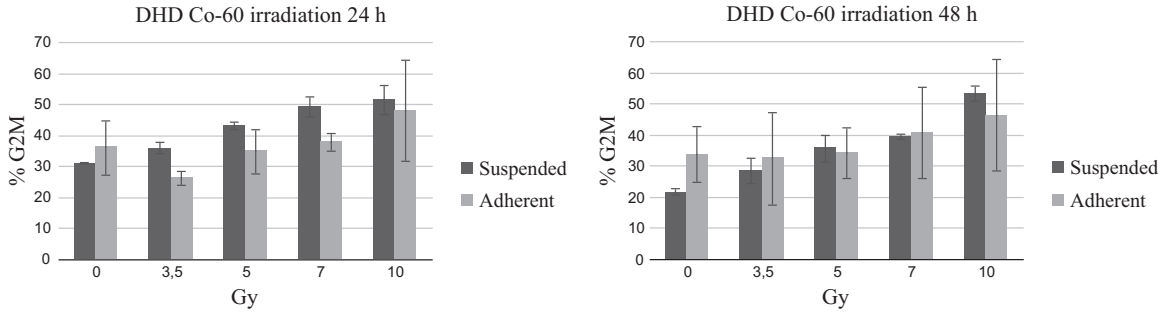

Fig. 2. Histograms of the percentage of G_2M cells 24 h (left) and 48 h (right) after ^{60}Co γ-ray 3.5, 5, 7 and 10 Gy doses: values of DHD cells exposed to radiation in suspension are compared with those of adherent exposed cells. G_2M of non irradiated control samples are also reported and compared.

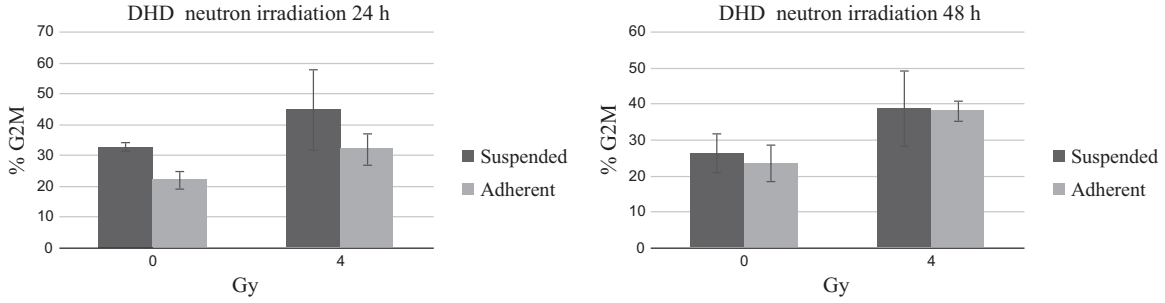

Fig. 3. Histograms of the percentage of G_2M cells 24 h (left) and 48 h (right) after 4 Gy thermal neutron irradiation: values of DHD cells exposed to radiation in suspension are compared with those of adherent exposed cells. G_2M of non irradiated control samples are also reported and compared.

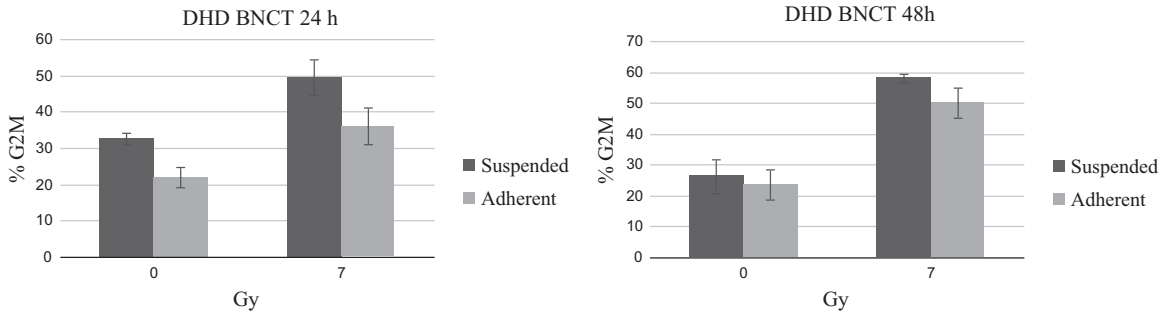

Fig. 4. Histograms of the percentage of G_2M cells 24 h (left) and 48 h (right) following 7 Gy BNCT treatment: values of DHD cells exposed when in suspension are compared with those of adherent irradiated cells. G_2M of non irradiated control samples are also reported and compared.

determined in order to avoid complete cell confluence at the end point. At the prefixed days of observation, irradiated and control cells were harvested by trypsinization, washed with PBS and, after accurate and gentle syringe clump dissociation, single cell suspensions were fixed in ethanol 70%. For DNA analyses, cells were stained with 50 μg/ml propidium iodide (PI) (Sigma, Italy). Measurements were performed on a PARTEC PAS II cytofluorimeter acquiring data in linear or log mode. Cell cycle phases were evaluated by the Flowmax dedicated software.

3. Results and discussion

3.1. Clonogenic cell survival

Fig. 1 shows the dose-related cell survival curves of DHD and UMR cells exposed to ^{60}Co, neutrons and BNCT, as cell monolayer adherent to the culture flask and as cell suspension inside vial. Each point represents the mean ± standard deviation of three repeated experiments. The dotted line represents the fit of irradiated cell suspensions while the continuous one that of adherent exposed cells.

Comparing experimental survival data, cells, irradiated in the adherent configuration, display a lower cell survival with respect to those irradiated when detached in suspension. Cell survival differences increase at high doses independently on the radiation type, clearly showing that adherent cells are more radiosensitive than the suspended ones.

Only in the case of neutron treatment, DHD cells show minimal survival differences, at least in the range of the tested doses.

Osteosarcoma UMR cells are more radiosensitive than coloncarcinoma DHD cells, independently on the radiation type and on the irradiation modality.

3.2. Radiation effects on cell cycle

DHD cells treated with ^{60}Co, were subjected to flow cytometric DNA analysis to follow the modifications induced to their cell cycle distribution. The G_2M percentages of suspended and adherent cells, 24 h and 48 h after irradiation, are reported and compared in Fig. 2.

Twenty-four hours after ^{60}Co irradiation, suspended cells show a dose dependant more marked G_2M increase with respect to adherent irradiated cells.

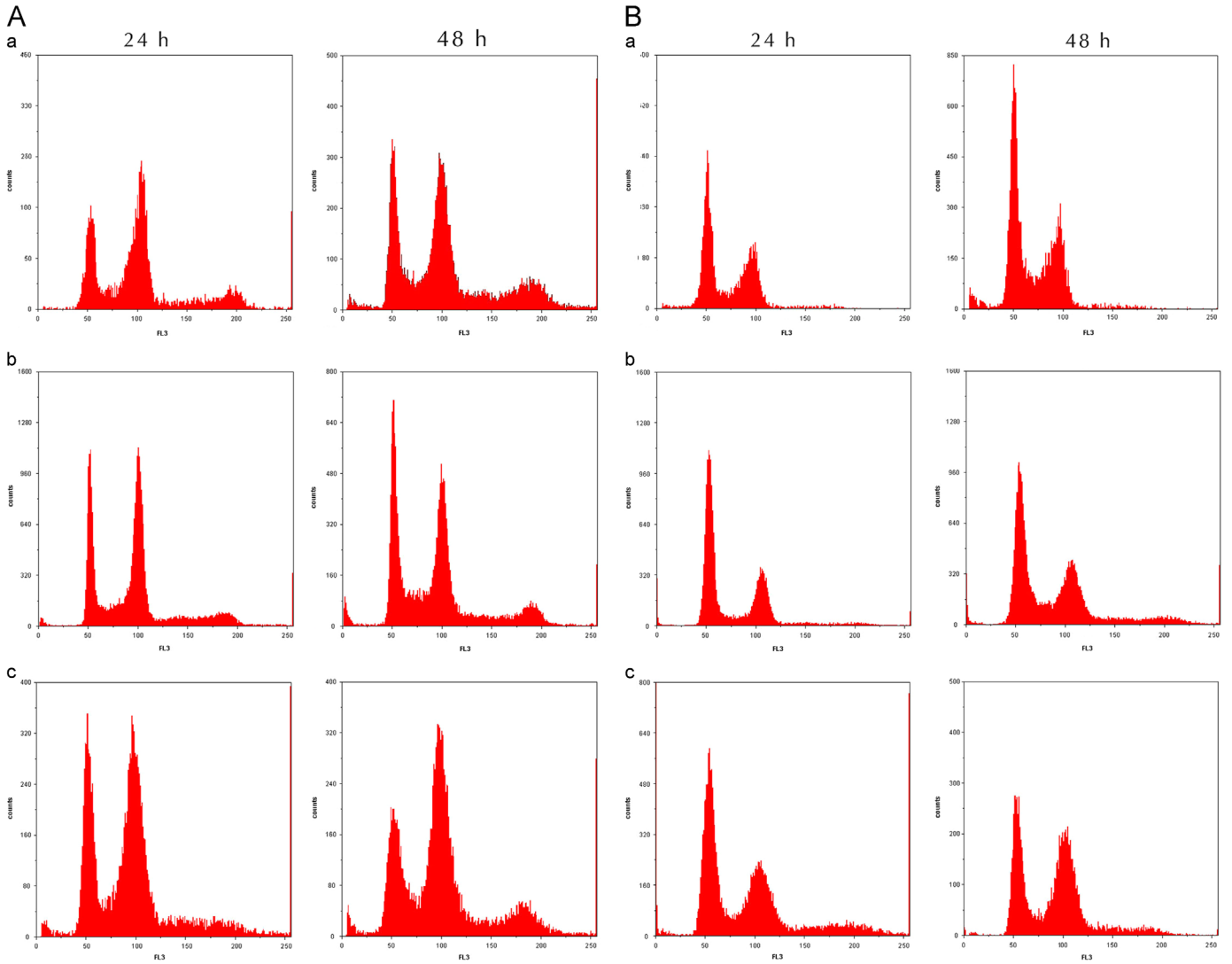

Fig. 5. (A) Examples of flow cytometric DNA histograms of suspended irradiated DHD cells, 24 h and 48 h after exposure to ^{60}Co γ-ray 7 Gy (a), thermal neutrons 4 Gy (b) and BNCT 7 Gy (c). The presence of an evident G_2M block and of tetraploid clones can be appreciated at all the tested conditions. (B) Examples of flow cytometric DNA histograms of adherent irradiated DHD cells, 24 h and 48 h after exposure to ^{60}Co γ-ray 7 Gy (a), thermal neutrons 4 Gy (b) and BNCT 7 Gy (c). The G_2M block and of tetraploid clones are less represented as compared to histograms of suspended cells.

Forty-eight hours after irradiation, an additional G_2M enhancement was observed only in case of suspended cells treated at 10 Gy dose. No differences of G_2M levels between suspended and adherent cells could be appreciated at all the other doses.

Suspended and adherent non irradiated control samples, 24 h after reseeding, show increased G_2M levels compared to those evaluated on cell samples at the time of irradiation (G_2M = 16%) and comparable to those observed at the lower radiation treatments (3.5, 5 Gy). After 48 h basal G_2M levels of suspended cells are almost completely restored, while those of adherent cells remain unchanged.

DNA cell cycle analyses performed on DHD cells exposed to neutrons only 4 Gy, show a behaviour comparable to that observed and above described following 7 Gy ^{60}Co dose delivering (Fig. 3).

In case of BNCT, 24 h post-treatment, an higher G_2M block in suspended than in adherent cells (49% *vs* 36%) can be noticed. An even more remarkable G_2M block that reaches comparable values in the two studied conditions (58% *vs* 50%) can be appreciated 48 h after irradiation (Fig. 4). The reported G2M percentages represent the mean values of three independent experiments.

Fig. 5A reports examples of flow cytometric DNA histograms performed on suspended cells, 24 h and 48 h after ^{60}Co γ-ray 7 Gy (a), neutrons only 4 Gy (b) and BNCT 7 Gy (c) irradiations. Fig. 5B shows histograms performed on adherent cells at the same modalities of suspended cells.

In addition to the above underlined differences of cell cycle distribution, the presence in histograms of suspended cells of a more marked tetraploid peak, both at 24 h and 48 h, can be highlighted. Moreover, analyses performed 5 and 7 days after BNCT treatment evidenced, the presence of multiclonal cell populations, with an even higher DNA content in case of cell suspensions. These findings are supported by the massive presence of multinucleated giant cells detected on reverse microscope flask observation (data not shown).

These results of cell survival and cell cycle perturbations evidence that radio-sensitivity differences between detached cells, irradiated in suspension, and those irradiated in the adherent configuration do exist, the suspended being the more resistant ones and those with a marked G_2M block. The higher G_2M block observed when cells were irradiated in the suspended set up might reflect the presence of a higher repair capacity that trigger cell survival increase. It has been reported that cell adhesion positively regulates the DNA-damage response to radiation (Lewis et al., 2002) and that G_2M accumulation after exposure to ionizing

radiation probably allows damaged cells to be repaired prior to mitosis (Kao et al., 2001), in accordance with our findings. In the past some authors studied cell survival on V79 cells irradiated as monolayer or spheroids. They observed that the survival of monolayer cells was lower than that of cells in spheroids. However the result was opposite when the time of plating was delayed, maintaining cells in the growing medium (Reddy and Lange, 1991; Gordon et al., 1990).

The analysis and comprehension of the molecular mechanisms related to the radio-sensitivity differences of suspended *vs* adherent irradiated cells, although extremely interesting, are not the aim of this study. Nevertheless, we verified that radio-sensitivity differences were not addressable to a different cell cycle distribution of cells at the time of radiation exposure (personal communication), while the possible role of hypoxia and of the suboptimal growing conditions are currently under investigation. The fact that cells that usually grow as adherent monolayers are maintained for some time detached in suspension, might influence their radio-sensitivity. Cells, unable to proliferate before their adhesion to the flask, may be able to mobilize resources for DNA repair that would make them more resistant. Those resources would be instead employed for cell cycle progression in case of adherent cells.

4. Conclusions

The main purpose of this comparative study was to verify the existence of radiosensitivity differences between adherent and suspended irradiated cells in order to optimize *in vitro* radiobiology experiments especially in the field of BNCT applied to cancer treatement.

The coloncarcinoma DHD and osteosarcoma UMR rat cell lines were exposed to ^{60}Co, neutrons only and BNCT irradiations, in two different set-ups: adherent to their culture flask and suspended inside vials after trypsinization.

Plating assay evidenced that suspended cells are more radioresistant than adherent cells to both low and high LET radiations.

Cell cycle of DHD cells, exposed to radiations in the two above described modalities were also evaluated. Histograms of DNA analyses showed a different cell cycle distributions of suspended *vs* adherent irradiated cells as proved by comparing G_2M values.

Therefore, the existence of a different response of suspended *vs* adherent irradiated cells is provided both by cell survival and cell cycle distribution results.

This radiobiologic aspect has crucial relevance in case of *in vitro* cell survival studies assessed by cloning assay. Although many papers have demonstrated a good correlation between cell survival assessed by cloning assay and that obtained by MTT test, at present clonogenic survival remains the “gold standard” for determining the radiosensitivity of cells *in vitro*. The methodology proposed and worldwide applied for cell survival evaluation by plating assay assesses that adherent cell cultures can be irradiated either before or after preparation of a single cell suspension. Conversely our findings suggest that the modality of cell exposure influences the cell radio-sensitivity. The literature related to *in vitro* BNCT cell survival, evaluated by cloning assay, does not differentiate data obtained on suspended *vs* adherent irradiated cells. Nevertheless optimization of the exposure condition for *in vitro* radiobiological experiments has recently been underlined and it is mandatory in order to compare inter and intra-laboratory results. Based on the work performed with these two cell lines, our indication is that the adherent configuration should be preferred, in order to maintain the cells in their natural environment, thus representing as close as possible the *in vivo* behaviour, considering the limitations of the *in vitro* experiment.

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