



## Evaluation of the cytotoxicity and the genotoxicity induced by $\alpha$ radiation in an A549 cell line

Ana Belchior<sup>a,b,\*</sup>, Octávia Monteiro Gil<sup>a</sup>, Pedro Almeida<sup>b</sup>, Pedro Vaz<sup>a</sup>

<sup>a</sup>Instituto Tecnológico e Nuclear, Estrada Nacional n° 10, 2686-953 Sacavém, Portugal

<sup>b</sup>Universidade de Lisboa, Instituto de Biofísica e Engenharia Biomédica, Campo Grande, 1749-016 Lisboa, Portugal

### ARTICLE INFO

#### Article history:

Received 16 March 2011

Accepted 9 April 2011

#### Keywords:

Alpha radiation

Biological indicators of exposure

Cytogenetics untargeted effects

### ABSTRACT

Exposure to radon and its progenies represents one of the greatest risks of ionizing radiation from natural sources. Nowadays, these risks are assessed by the extrapolation of biological effects observed from epidemiological data. In the present study, we made a dose response curve, to evaluate the *in vitro* response of A549 human lung cells to  $\alpha$ -radiation resulting from the decay of a  $^{210}\text{Po}$  source, evaluated by the cytokinesis blocked micronuclei assay. The clonogenic assay was used to measure the survival cell fraction. As expected, the results revealed an increase of cellular damage with increased doses made evident from the increased number of micronuclei (MN) per binucleated cell (BN). Besides this study involving the biological effects induced by direct irradiation, and due to the fact that radiation-induced genomic instability is thought to be an early event in radiation carcinogenesis, we analyzed the genomic instability in early and delayed untargeted effects, by using the medium transfer technique. The obtained results show that unirradiated cells exposed to irradiated medium reveal a higher cellular damage in earlier effects when compared to the delayed effects. The obtained results may provide clues for the biosimetric determination of radon dose to airway cells at cumulative exposures.

© 2011 Elsevier Ltd. All rights reserved.

### 1. Introduction

It is rather consensual in the scientific community the need to understand the biological risks attributable to low doses of ionizing radiation (Sachs and Brenner, 2005 and Prasad et al., 2004). In addition, selecting the most reliable dose–effect relationship based on biological data is also considered of paramount importance. One of the most successful approaches to model these effects is the Linear Non Threshold (LNT) model (Tubiana et al., 2006; Brenner and Sachs, 2006). The epidemiological basis underlying this approach allowed it to become an “anchor point”, which was subsequently used to obtain a dose–effect relationship for a given dose value. Recent developments suggest that cellular response to low doses of ionizing radiation cannot necessarily be directly extrapolated from higher doses (Mullenders et al., 2009; Averbeck, 2009). One of the aims of this study was to perform a dose response curve for the evaluation of A549 human cell line response to several doses of  $\alpha$ -radiation. We use a human lung cell line and  $\alpha$ -radiation emitted by a monoenergetic alpha source with the same energy of radon, 5.297 MeV, in order to quantify the cellular damage that could be observed *in vivo* by radon inhalation. Radon exposure is

\* Corresponding author. Instituto Tecnológico e Nuclear, Estrada Nacional n° 10, 2686-953 Sacavém, Portugal.

E-mail address: [anabelchior@itn.pt](mailto:anabelchior@itn.pt) (A. Belchior).

a human health concern, and it is demonstrated that very low doses of high LET  $\alpha$ -particle irradiation initiate deleterious genetic consequences in both irradiated and non-irradiated bystander cells (Bowler et al., 2006). One consequence, radiation-induced genomic instability, is often assessed by measuring delayed cellular damage.

Besides, the LNT model's has been challenged also by studies where cellular damage arise in cells that were not exposed to radiation (Suzuki et al., 2004; Nagasawa et al., 2003). These, so called, untargeted effects were demonstrated by media transfer (radiation-induced genomic instability) or in cells that have communicated with irradiated cells (radiation induced bystander effects). Despite this exposure, after quantifying the cellular response and survival fraction induced by direct irradiation of A549 human lung adenocarcinoma cell line using the cytokinesis blocked micronucleus assay and the clonogenic assay, respectively, we study as well if the untargeted effects are inducible using the same cell line and type of radiation and moreover if genomic instability is observed.

### 2. Materials and methods

#### 2.1. Cell line and reagents

Cell line and main reagents used in this study included a human lung adenocarcinoma cell line A549 (gently given by University of

Porto, Portugal), Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin solution cytochalasin B (Sigma–Aldrich, USA), Thiazolyl Blue Tetrazolium Bromide (Alfa Aescar, Germany), trypsin and other reagents such as Giemsa dye (Sigma–Aldrich, USA), methanol, acetic acid.

## 2.2. Cell culture and cell irradiation

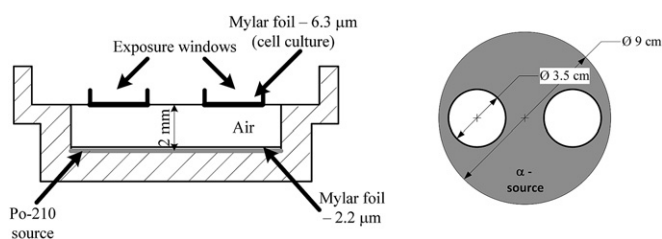
A549 cells were cultured at 37 °C with 5% CO<sub>2</sub> in DMEM<sup>1</sup> medium containing 10% FBS and 1% penicillin-streptomycin solution. Log-phase cells were seeded onto 3.5 cm culture dishes with 6.3 μm of Mylar<sup>®</sup> base 24 h before irradiation. Cells at exponential growth were acutely exposed to the following doses; 0, 50, 100, 500, 1000, 1500 and 2000 mGy, by using a monoenergetic <sup>210</sup>Po source developed by Szabó et al., (2002), characterized by emitting 5.297 MeV α-particles with a LET of 156 keV/μm (Belchior et al., 2010). The cell cultures are positioned at the exposure windows placed a few millimeters above α-particles source, as shown in Fig. 1.

## 2.3. The cytokinesis blocked micronucleus assay

A population of approximately 1 × 10<sup>5</sup> cells was cultured in 3 mL of DMEM<sup>2</sup> medium supplemented with 10% of fetal bovine serum and 1% of Penicillin-Streptomycin Solution. After 24 h of incubation, different cell cultures were irradiated with dose values referred above. Control cultures were submitted to the same experimental conditions but not irradiated. At 44 h of incubation, cytochalasin B with a concentration of 2 mg/ml was added to the culture medium to inhibit cytokinesis, and allow identification of binucleated cells. Cells are generally incubated with cytochalasin B for approximately 1–2 cell cycle in order to gather the majority of the cells at binucleated state. At the end of incubation cells were harvested by centrifugation and submitted to a mild hypotonic shock to enlarge the cytoplasm of the cell. The cells were then smeared onto clean glass slides, allowed to dry, fixed with methanol: acid acetic and finally stained with Giemsa 4%. MNs were identified according to the criteria previously published by Fenech (Fenech, 2000). The frequency of binucleated cells containing one or more MN was also determined.

## 2.4. The clonogenic assay

The cells were cultured and irradiated in the same conditions as Section 2.3. The cells were then seeded in an appropriate dilution (±200 cells per culture) within Petri dishes for cell culture and incubate for more 10 days. Colonies of cells formed were subsequently counted. The Plating Efficiency (PE) ratio is defined as:



**Fig. 1.** Cross-section (left) and top view (right) of the irradiation device. The α-particles cross a membrane of 2.2 μm of Mylar before reach the 2 mm air layer, and then reach the cell monolayer after cross the 6.3 μm of Mylar where cells are cultured.

PE values were determined using the non-irradiated cells. The cell surviving fraction (SF) is the number of cell colonies that arise after irradiation of cells, expressed in terms of PE.

## 2.5. Early and delayed untargeted effects of α particles in A549 cells

To perform this study, the cells were exposed to 1000 mGy, and the following experiments were performed:

*Experiment I* – Irradiated cells, collected in fresh medium, and re-cultured with a concentration of 1 × 10<sup>4</sup> and 1 × 10<sup>2</sup> for early and delayed cytogenetic studies, respectively.

*Experiment II* – Cells were irradiated on replicate Mylar dishes and collected into fresh medium as in the case of Group I. These cells were collected by centrifugation at 1200 rpm for 5 min; the medium was filtered using a 2.2 μm membrane filter (Millipore) and transferred to non-irradiated cultures for cytogenetic studies after 2 and 5 days of treatment.

*Experiment III* – The irradiated cells collected from Group II were cultured with the same concentrations as in experiment I for cytogenetic studies 2 and 5 days after irradiation. In this experiment any radiation induced bystander effect is minimized with respect to the experimental conditions in experiment I due to the cell re-suspension in fresh medium.

Cytochalasin B was added to the cultures, 2 and 5 days after irradiation, for early and delayed studies, respectively.

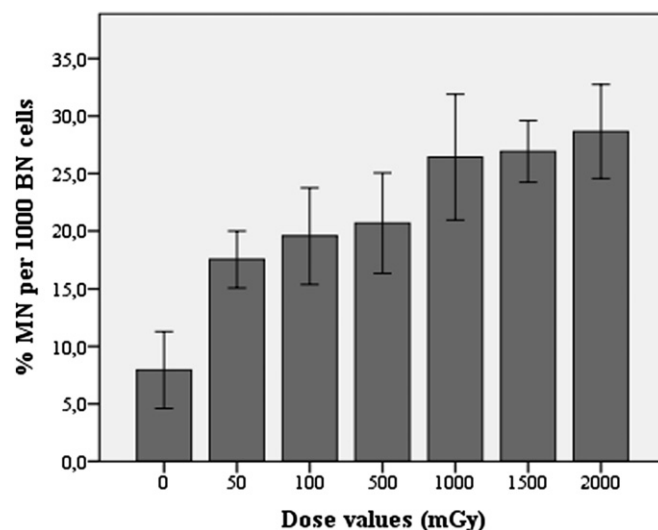
## 2.6. Statistical analysis

All values are means ± SEM of 3 independent experiences. The significance of the differences between means was evaluated by the Student's *t*-test. Probabilities of *p* < 0.05 and <0.01 were taken as significant and very significant differences, respectively.

## 3. Results and discussion

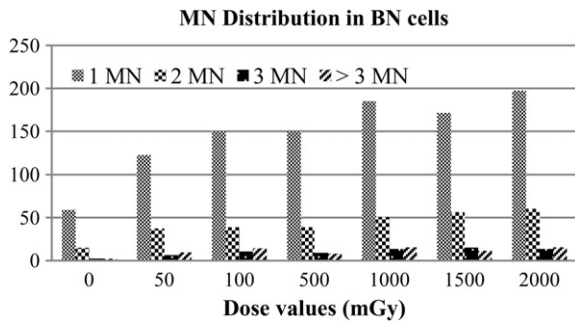
### 3.1. Cellular damage

Fig. 2 depicts the number of MN present in 1000 BN cells scored and Fig. 3 the MN distribution in BN cells. From the analysis of Fig. 2,



**Fig. 2.** Dose response curve. Number of MN in 1000 BN cells (mean ± SEM) of 3 independent experiments. By analyzing the results, it can be summed up that there is a significant increase of MN per 1000 BN cells when the result of each dose is compared with the control (non-irradiated cells). This indicates that the cellular damage increase with dose values, as each MN represents a specific lesion.

<sup>1</sup> DMEM – Dulbecco's Modified Eagle's Medium, Sigma Aldrich, USA.



**Fig. 3.** MN distribution in BN cells. 1 MN, 2 MN, 3 MN and >3 MN means that BN cells contain one, two, three and more than three MN. The obtained results show that genetic lesion results essentially in BN cells with only one MN. As dose values increase, the frequency of 2 MN becomes more evident. By the appearance of more than one MN, one can predict that higher doses are more damaged to the cell.

it can be inferred that the genetic lesion induced by  $\alpha$ -particles in A549 cells increase with increasing dose values, which is clear from the increase in the number of MN in BN cells. Fig. 3 shows that genetic lesion results essentially in BN cells with only one MN. As dose increases, the frequency of 2 MN or more also increases in the BN cells scored, which could indicate that higher dose values result in a different genetic lesion when compared to lower ones.

3.2. Clonogenic assay

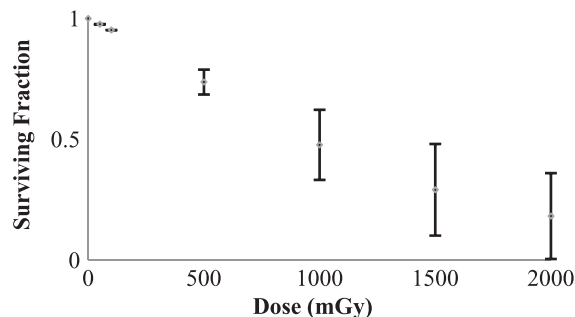
Fig. 4 shows the surviving fraction for different values of radiation doses.

As can be seen from Fig. 4, there is a trend for a decreasing of the cell surviving fraction as the dose values increase. This decrease is significant for all irradiated samples when compared to the unirradiated controls, for doses up to 100 mGy,  $p < 0.01$  and up to 2000 mGy,  $p < 0.05$ .

3.3. Untargeted effects – early and delayed effects

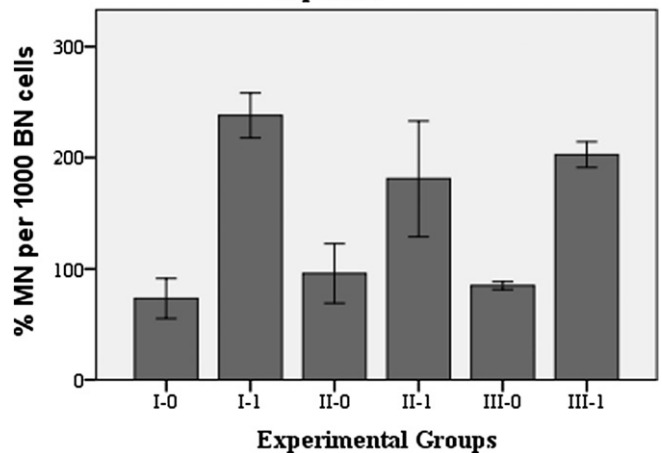
Figs. 5 and 6 show the number of MN in 1000 BN cells, 2 (early effects) and 5 (delayed effects) days after irradiation, respectively.

Radiation exposure resulted in a significant increase in the frequency of MN per BN cell when compared to the control situation (non-irradiated cells). As expected, in group I this increase is more evident when compared with group III, because any expected radiation induced damage/bystander factor was removed from group III by centrifugation of cells and re-cultured in fresh medium. Group II experiment also shows an evident increase in the frequency of MN in BN cells when compared to controls. As mentioned before, a cytogenetic analysis (cytokinesis blocked



**Fig. 4.** Cell survival curve in A549 cells. Each data point represents the mean  $\pm$  sd of the clonogenic survivals from three culture plates.

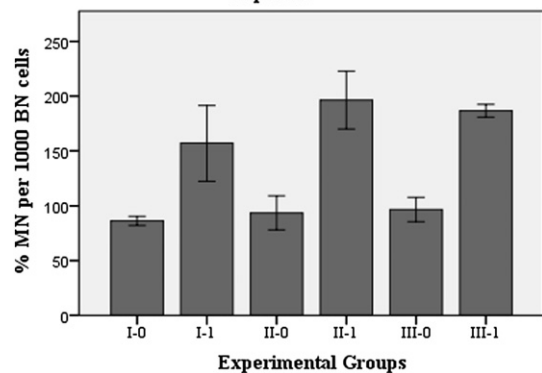
Number of MN per 1000 BN cells - after 2 days of exposure



**Fig. 5.** Number of MN per 1000 BN cells (%MNBN) at 2 days post-irradiation for groups I to III. The unirradiated controls are marked as I(to III)-0 and from I to III are the results obtained for irradiated cultures. Data represent means of 3 independent experiments,  $\pm$  S.E.M. A significantly increase of %MNBN was observed in groups I and III ( $p < 0.005$ ) and in group II ( $p < 0.05$ ), when compared to their own controls.

micronucleus assay) was performed 5 days after irradiation in order to study the delayed cellular damage. The delayed response of cells to radiation is similar to the earlier response, with respect to the increase of MN in exposed cells (when compared to controls). However, in the results from Group II, in both delayed and earlier responses, one can notice an increase of MN in the delayed response case. As said before, the study of delayed response to radiation is important to assess genomic instability after irradiation. Genomic instability is associated with the loss of cell cycle control and to alterations in the DNA repairs processes, and can give rise to a variety of observable cellular changes (Bowler et al., 2006). By analyzing the data displayed in Figs. 5 and 6 it becomes evident that the genomic instability is more apparent 5 days after cell irradiation than after only 2 days. Groups II and III in Fig. 5 show a decrease in the frequency of MN when compared to the corresponding results depicted in Fig. 6. This can suggest that the DNA repair mechanism could fix the genetic lesion induced by radiation or that cells go through apoptosis and/or necrosis processes (Hickman, 2002).

Number of MN per 1000 BN cells - after 5 days of exposure



**Fig. 6.** Number of MN per 1000 BN cells (%MNBN) at 5 days post-irradiation for groups I to III. The unirradiated controls are marked as I(to III)-0 and from I to III are the results obtained for irradiated cultures. Data represent means of 3 independent experiments,  $\pm$  S.E.M. At day 5, the %MNBN was significantly increased in groups I, II and III ( $p < 0.05$ ,  $p < 0.01$  and  $p < 0.1$ , respectively).

#### 4. Conclusion

Recent data has revealed the complexity and efficacy of biological defense mechanisms against genotoxic agents (physical and chemical) at cell (DNA repair and apoptosis), tissue (role of neighboring cells), and whole body (immunosurveillance) level, in which ionizing radiation is likely to induce, at different levels depending on cells, apoptotic responses, which are the consequence of intra- and inter-cellular signaling. However, ionizing radiation can also induce mutations, which interfere with apoptosis and consequently permit the survival of damaged cells. In turn, mutation constitutes one of the steps of carcinogenesis. Concerning the effects of direct radiation exposure, the obtained results reveal an increase in the number of cellular lesions as a consequence of increasing absorbed dose values, as quantified by MN number after cell irradiation. The results obtained also show that the more frequent lesion observed after cell absorbed dose ranging from 50 to 2000 mGy is the presence of 1 MN per BN cell. For higher doses it is evident an increase in the frequency of 2 or more MN per BN cell. Micronuclei are fragments of genetic material that contain either acentric fragments (resulting from DNA breaks), whole chromosomes, or complex rearrangements that are unable to properly attach and be pulled to the poles by the mitotic spindle. So, although this study is not able to identify the occurred genetic lesions, it presents clear data showing that higher absorbed doses by cells result in more DNA lesions. These lesions could, in turn, incite a biological mechanism unsuitable to be repaired and consequently the death of the cell. In fact, it is known that low dose absorption and irradiation from low dose rate radiation may be able to induce significant apoptosis. Moreover, apoptosis may be one of the mechanisms by which low dose absorption causes growth inhibition (Joniani et al., 2000; Dewey et al., 1995 and Hickman, 2002), which challenges the LNT model's validity. Moreover, concerning cell killing the obtained results reveal a significant decreasing with dose increasing, due to a decrease in the surviving fraction with dose. This study and associate findings must be complemented by more accurate quantification of cell survival in order to better understand the results obtained.

Previous studies on biological untargeted effects of irradiation using medium transfer have shown that medium from irradiated cells can induce bystander effects in non-irradiated cells (Azzam et al., 2002; Hu et al., 2006; Lyng et al., 2000). In this context, the aim of our work was to study the influence of the irradiated medium that induce cellular damage in unirradiated cells, in early and delayed effects. At day 2 after cell irradiation, a more evident increase of the number of MN per BN cell in group I was observed when compared to group III. This is due to the fact that any expected radiation induced damage/bystander factor originating from irradiated cell cultures was removed from group III by centrifugation of cells and re-culture in fresh medium. Group II shows a clear increase in the frequency of MN in BN cells when compared to the non-irradiated control. In order to study delayed cellular damage, a cytogenetic analysis was performed 5 days after irradiation. The delayed response of cells to radiation is similar to the response obtained 2 days after irradiation, in which concerns the increase in the number of MN in exposed cells when compared to controls. However, in Group II, the comparison of results obtained for delayed and earlier responses, one can notice an increase in the number of MN in the delayed response. Data obtained in other cell lines show that the induction of cellular damage in bystander cells persist in time, probably as a consequence of the formation of bystander factors that themselves generate reactive oxygen species ROS,

leading to a self-sustaining system responsible for delayed effects (Yang et al., 2005; Sokolov et al., 2005). Our results are in agreement with these evidences and add additional proof to the existence of a bystander signal that may be responsible for cellular damage late after cell irradiation. The obtained results may provide clues for the biodosimetric determination of radon dose to airway cells at cumulative exposures.

#### Acknowledgments

The work was partially supported by FCT/MCTES grant No. SFRH/BD/42172/2007 and by ITN. A special thanks to Dr. Imre Balashazy (KFKI, Hungary) who has kindly provided the device for cell irradiation and the  $^{210}\text{Po}$  sources used in this work and to Prof. Luis Peralta (FC-UL) who helped with the measurement of alpha particle energy spectrum performed in the Faculty of Sciences, University of Lisbon.

#### References

- Averbeck, D., 2009. Does scientific evidence support a change from the LNT model for low-dose radiation risk extrapolation? *Health Phys.* 97, 493–504.
- Azzam, E.I., Toledo, S.M., Spitz, D.R., Little, J.B., 2002. Oxidative metabolism modulates signal transduction and micronucleus formation in bystander cells from alpha-particle-irradiated normal human fibroblast cultures. *Cancer Res.* 62, 5436–5442.
- Brenner, D., Sachs, R., 2006. Estimating radiation-induced cancer risks at very low doses: rationale for using a linear no-threshold approach. *Radiat. Environ. Biophys.* 44, 253–325.
- Belchior, A., Peralta, L., Almeida, P., Vaz, P., 2010. Calibration of an alpha particle irradiator for in vitro cells irradiation. *Int. J. Low Radiat.* 7 (no. 6).
- Bowler, D., Moore, S., Macdonald, D., Smyth, S., Clapham, P., Kadhim, M., 2006. Bystander-mediated genomic instability after high LET radiation in murine primary haemopoietic stem cells. *Mutat. Res.* 597, 50–61.
- Dewey, W., Ling, C., Meyn, R.E., 1995. Radiation-Induced apoptosis: relevance to radiotherapy. *J. Radiat. Oncol. Biol. Phys.* 33 (No. 4), 781–796.
- Fenech, M., 2000. The in vitro micronucleus technique. *Mutat. Res.* 455, 81–95.
- Hickman, J.A., 2002. Apoptosis and tumorigenesis. *Cur Opin. Cell Biol.* 12, 67–72.
- Hu, B., Wu, L., Han, W., Zhang, L., Chen, S., Xu, S., Hei, T.K., Yu, Z., 2006. The time and spatial effects of bystander response in mammalian cells induced by low dose radiation. *Carcinogenesis* 27, 245–251.
- Joniani, H.M., Eriksson, D., Sheikholvaezin, A., Johansson, A., Lofroth, P., Johansson, L., Stigbrand, T., 2000. Apoptosis induced by low dose and low dose rate radiation. In: 8th Conference of Radioimmunodetection and Radioimmunotherapy of Cancer, Princeton, New Jersey.
- Lyng, F.M., Seymour, C.B., Mothersill, C., 2000. Production of a signal by irradiated cells which leads to a response in unirradiated cells characteristic of initiation of apoptosis. *Br. J. Cancer* 83, 1223–1230.
- Mullenders, L., Atkinson, M., Paretzke, L., Sabatier, L., Bouffler, S., 2009. Assessing cancer risks of low dose radiation. *Nat. Rev. Cancer*, 596–604.
- Nagasawa, H., Huo, L., Little, J.B., 2003. Increased bystander mutagenic effect in DNA double-strand break repair-deficient mammalian cells. *Int. J. Radiat. Biol.* 79, 35–41.
- Prasad, K.N., Cole, W.C., Hasse, G.M., 2004. Health risks of low dose ionizing radiation in humans: a review. *Exp. Biol. Med.* 229, 378–382.
- Sachs, R., Brenner, D., 2005. Solid tumor risks after high doses of ionizing radiation. In: Proceedings of the National Academy of Science of the United States of America.
- Sokolov, M.V., Smilenov, L.B., Hall, E.J., Panyutin, I.G., Bonner, W.M., Sedelnikova, O.A., 2005. Ionizing radiation induces DNA double-strand breaks in bystander primary human fibroblasts. *Oncogene* 24, 7257–7265.
- Suzuki, M., Zhou, H., Geard, C.R., Hei, T.K., 2004. Effect of medium on chromatin damage in bystander mammalian cells. *Radiat. Res.* 162, 264–269.
- Szabó, J., Fehér, J., Pálfalvi, J., Balásházy, I., Dám, A.M., Polonyi, I., Bogdándi, E.N., 2002. In vitro cell irradiation systems based on  $^{210}\text{Po}$  alpha source: construction and characterization. *Radiat. Meas.* 35, 575–578.
- Tubiana, M., Aurengo, A., Averbeck, D., Masse, R., 2006. The debate on the use of a linear no threshold for assessing the effects of low doses. *J. Radiol. Prot.* 26, 317–324.
- Yang, H., Assad, N., Held, K.D., 2005. Medium-mediated intercellular communication is involved in bystander responses of X-ray – irradiated normal human fibroblasts. *Oncogene* 24, 2096–2103.