QUANTIFICATION OF DNA DAMAGE IN HUMAN LYMPHOCYTES BY COMET ASSAY, DURING *IN VITRO* AGEING IN THE PRESENCE OF RADON

DANIELA CIORBA[*], *V. MORARIU*[**], *C. COSMA*[*], *SILVIA NEAMȚU*[**], *CORINA CUCEU*[*]

[*] Environmental Science Faculty, "Babeş-Bolyai" University, 30, Fântânele St, Cluj-Napoca Romania, e-mail: ciorbad @yahoo.com
[**] National R & D Institute of Isotopic & Molecular Technologies, Cluj-Napoca, Romania

Abstract. *In vitro* ageing of human lymphocytes is expected in cell cultures at a temperature of 23 °C, while the necessary condition for cell division is 37 °C. The number of lesions in stage 4 during 3 days of aging was found to increase. Also an obvious decrease of lesions in stage 1 was noticed. This has a significant influence in the final lesion score, and tail factor. When the aging process of lymphocytes took place in radon atmosphere, a significant acceleration was noticed (while the temperature of 23 °C remains unchanged). The increase of the aging process is due to a significant acceleration of the swallowing process. We further show that use of the Colin's formula to quantify induced DNA lesions during aging *in vitro* is not sufficient because we cannot see a linear dependence between estimated parameters: score lesions, tail factor and the exposure time. An additional argument to this was brought also by the aging process during radon exposure. Even if the Comet assay is suitable for DNA damage, it must be improved by cell viability studies and cells counting.

Key words: *in vitro* ageing, lymphocytes, radon, Comet assay, interfering, DNA lesions.

INTRODUCTION

Currently, human health risk estimates for the effects associated with radiation exposures are based primarily on the view that the detrimental effects of irradiation occur only in irradiated cells. The single cell gel electrophoresis (SCGE) or comet assay (CA) has been considered a rapid, simple and sensitive technique for measuring DNA damage [17, 26]. The alkaline version of the method is widespread and frequently used for detecting the genetic damage induced by different genotoxic agents such as radiation [12, 22, 28].

Many studies were done concerning the irradiation of lymphocytes [4, 16, 24]. Using the radon exposure, the cytogenetical damage in human blood lymphocytes was reported recently for concentrations similar with those used in this experiment [15, 27].

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From our first studies, we observed the increase of cell's aging after the human blood exposure in radon atmosphere [8–11].

The novelty of this study is that we monitored only lymphocytes in culture from integral blood. The DNA damages were observed comparatively for the ageing process at the temperature of 22°C and also for the exposure at high concentrations of radon whose genotoxic potential is recognized [13]. It is a study of toxicity *in vitro* which, differently from the other toxicology studies for individual doses, uses a kinetic model. The ageing of lymphocytes is continuous, and the radon concentration increases with about 4 times compared to the control value, during of the 3 days of exposure (72 hours).

MATERIALS AND METHODS

IN VITRO IRRADIATION SYSTEM USING PITCHBLENDE ORE

Pitchblende ore is a uranium ore (UO_3, U_2O_5, U_3O_8) (Fig. 1). The natural airborne radioactivity results by disintegration of "parent-progeny", so-called "radon-thoron" (RnTn). These radioactive isotopes are the main contributors to the dose from natural radiation sources. The irradiation doses inside of experimental box after 24 hours was 89.15 kBq/m^3; 162.6 kBq/m^3 after 48 hours, respectively 212.9 kBq/m^3 after 72 hours (Table 1).

Table 1

The irradiation doses during *in vitro* exposure. Temperature = 22 °C

Time of exposure (hours)	Radon concentrations (kBqm^{-3})
24	89.15
48	162.6
72	212.9
96	220

10 mL of blood is taken from a healthy donor by venipuncture. Lymphocytes are separated using the density gradient protocol, on Histopaque-1077. Briefly, blood is diluted 1:1 with PBS and layered over 600 μL Histopaque and centrifuged at 800 × g for 20 minutes. The 'buffy' coat, an opalescent layer containing mononuclear cells, is aspirated into 3–5 mL of PBS and centrifuged at 250 × g for 10 minutes to pellet the lymphocytes. The pellet is suspended in 1 mL of (RPMI + 15% fetal serum, + 10% glutamine + antibiotics) in test tubes. Aliquots of 2 × 10^6 cells per 100 μL of medium are taken for each dose of the test material.

Aliquots containing lymphocytes were diluted with PBS 1:10 and tubes are inverted to mix the cells and test material in order to obtain a homogeneous suspension. The DNA damage has been detected in isolated lymphocytes obtained from 5 healthy donors, and then it was exposed to ionizing radiations emitted to pitchblende ore, Rn (Fig. 1)

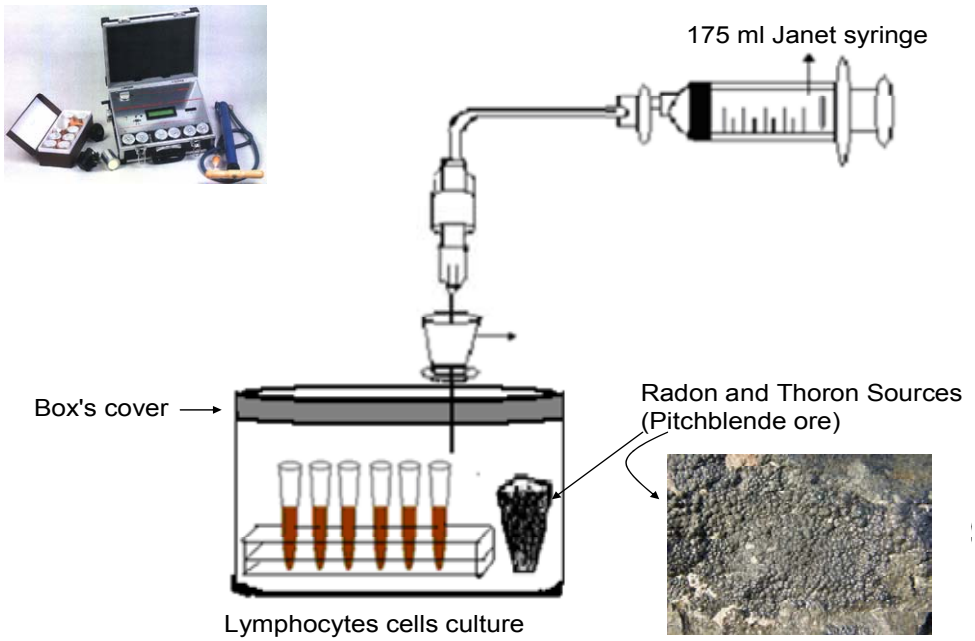

Fig. 1. Assembly for *in vitro* irradiation, using pitchblende ore.

The working protocol used to detect DNA damage was in accord with ITRC: THE SCGE/ COMET ASSAY PROTOCOL modified after Brie *et al*. [6].

EVALUATION OF DNA DAMAGE

The overall structure resembles a comet with a circular head, corresponding to the undamaged DNA that remains in the cavity and a tail of damaged DNA (Fig. 2). Visual and computerized image analyses of DNA damage were carried out based on perceived comet tail length migration and relative proportion of DNA in the comet tail (Fig. 2). Etidium Bromide-stained nucleoids were examined at 40× objective with a Axioplan, Zeiss fluorescence microscope, at 460 nm. Individual comet images were captured for digitization with a CCD camera attached to the microscope.

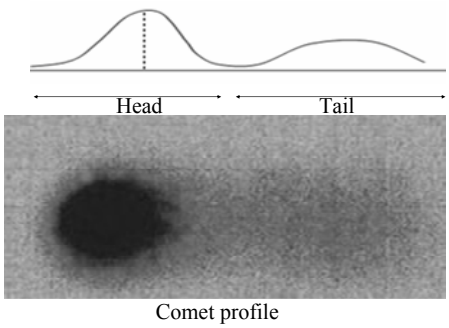

Fig. 2. The overall structure of the COMET.

One hundred randomly selected no overlapping cells on each control or sample slide after 24, 48, 72 hours were scored visually. The cells were classified as belonging to one of the five predefined classes according to tail intensity and given a value of 0, 1, 2, 3 or 4 from undamaged 0 to maximally damaged 4 (Fig. 3).

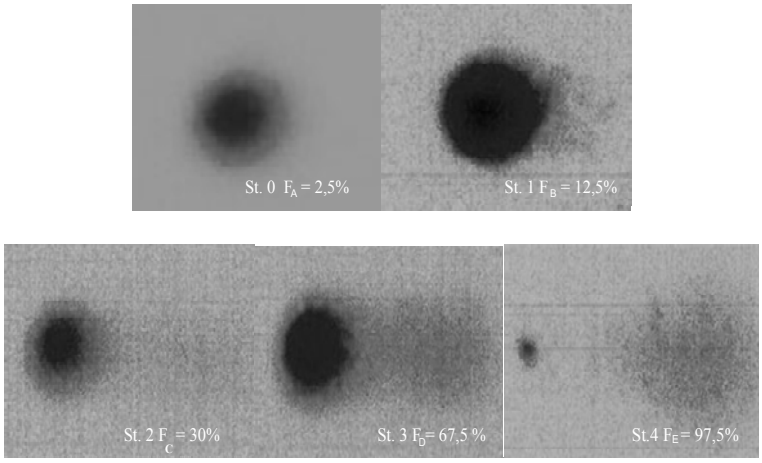

Fig. 3. Visual score of predefined comet classes according to tail intensity (cells stadiums of 0, 1, 2, 3 or 4 correspond from undamaged 0 to maximally damaged 4).

Scoring for DNA damage – score lesion (SL) = the sum of relative units, (UR), means the product between cells number finding in a special stadium and the number of comet class.

$$SL\ (UR) = A0 + B1 + C2 + D3 + E4 \quad (1)$$

where A, B, C, D, E, = number of cells in 0, 1, 2, 3, and 4 stadium.

A total damage score for each slide was derived by multiplying the number of cells assigned to each grade of damage by the numerical value of the grade and summing over all grades (giving a maximum possible score of 400, corresponding to 100 cells at grade 4).

Tail factor (TF) represents a quantification parameter over 500 of studied cells, meaning the relative percent of DNA in tail.

$$TF(\%) = (A\ FA + B\ FB + C\ FC + D\ FD + E\ FE)/500 \quad (2)$$

where FA, FB, FC, FD, FE is the relative percent of DNA in tail.

RESULTS

DNA damage has been followed on 100 cells, for each subject, in every day of exposure.

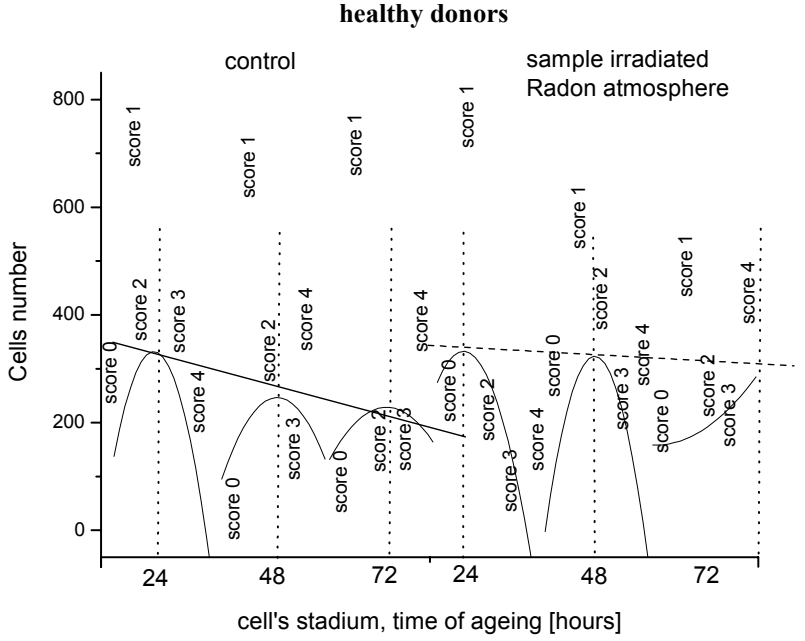

Fig. 4. Lymphocytes damage when cells culture ageing *in vitro* (control) and for radon exposure (sample). T = 22 °C. The best polynomial fit of the cells number for all stadiums.

The cells finding in one described stadium was comparatively represented for control and exposed sample to radon (Fig. 4).

In Table 2 are presented the results obtained after 72 hours of exposure.

Table 2

Effects after the irradiation with alpha particles; compared with control

24 hours of exposure	**48 hours** of exposure	**72 hours** of exposure
Significant increases only for cells with Score 1	Significant increases for cells with Score 2, with 123%;	Significant increases of cells with Score 2, 3 and 4.
Qualitative effect: transient to higher cell stadium, equivalent with the increase of DNA damage		

The maximum values for the irradiated samples in 72 h of ageing are indicated in Table 3.

Table 3

Ageing effect for irradiated sample; comparison between the days of exposure

After 24 hours, day 1	After 48 hours, day 2	After 72 hours, day 3
Maximum of distribution at 335.37	Maximum of distribution at 321.27 with 4.2% lower than those recorded at 24 hours.	Maximum of distribution at 241.11; with 33.24% lower than recorded in 48 hours.
Quantitative effect: Apparent decreases of cells damaged number after 72 hours of ageing, compared with 48 hours or 24 hours (Fig. 5).		

The same apparent decrease in time of cells damaged number has been observed for the control, but the decreasing is more accentuated (Fig. 5).

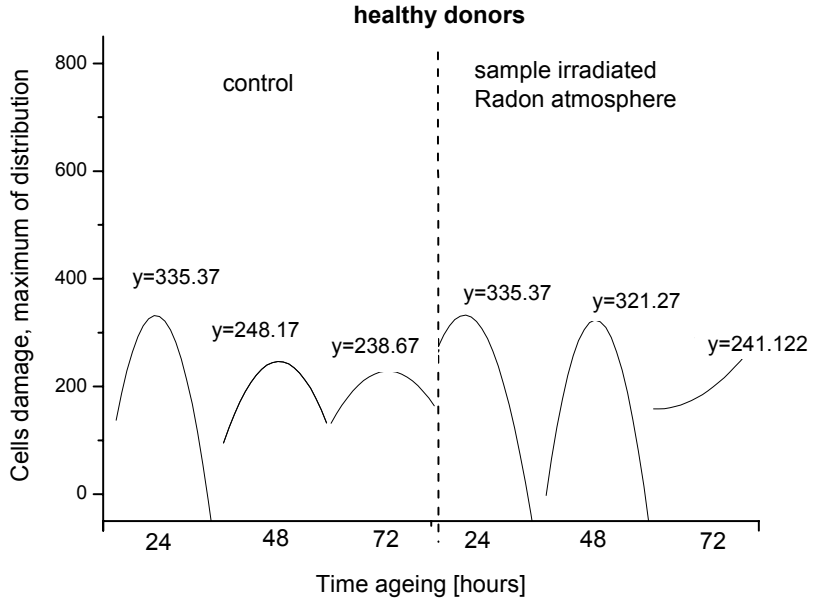

Fig. 5. The polynomial fit of the kinetic process of *in vitro* lymphocytes ageing, representation of distribution maximum (control) and radon exposure (sample), for each day of exposure. T=22 °C.

Using only the points registered for the distribution maximum after 24 h, 48 h, and 72 h it was obtained a single overall distribution for control and sample (Fig. 6).

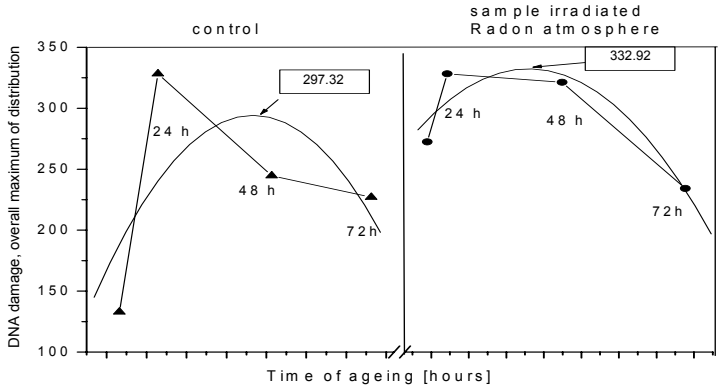

Fig. 6. Variation of cells damaged number for the control and sample, overall kinetic process are represented using the polynomial fit. T=22 °C.

According to our results, the maximum value of this curve is higher for the irradiated sample than for the control. Quantitatively, the number of cells damaged is higher after irradiation (Fig. 6). By the other way, DNA damage may be expressed through the lesions score, SL and tail factor, TF, applying the Colin's formula (Fig. 7) [16]. This formula is usually used for the study of the toxicity in radiation. SL and TF are very low, near zero at start point (Fig. 7).

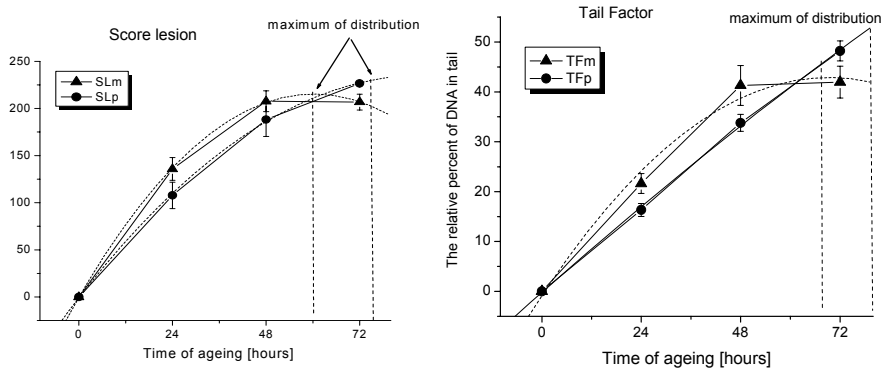

Fig. 7. Evaluation of radon toxicity during the lymphocytes ageing by score lesions and tail factor overlap of the best polynomial fitting for induced effects. T = 22 °C. Legends: SLm – score lesion control, SLp – score lesion sample (irradiated cells); TFm – tail factor control, TFp – tail factor sample (irradiated cells).

SL and TF revealed the increases of DNA damage with ageing, but the appearance of distribution maximum is delayed and higher for the irradiated sample (Fig. 7).

Apparently the decrease of the number of cells damaged after 72 hours of ageing, comparatively with 48 or 24 hours (Fig. 5), corresponds with a shift to a higher cell's stadium, equivalent with increases of DNA damage (Fig. 4, Table 2) and with an increased rate for both SL and TF (Fig. 7). If we observe our process comparatively for the control and sample only for 48 hours, the final conclusion that the induced toxicity after the irradiation is not so important as the ageing process could be wrong. Taking into account the observed delay in the appearance of maximum, and the maximum differences (Fig. 7), according also to Figure 6, we can say that the toxicity increases in the presence of radon exposure.

DISCUSSION

Like environmental genotoxic agents, the radon gases induce lesions in cells, which may be manifested at any level of organization of the genetic material, i.e., the chromosome, the gene, or the primary DNA level. Comet assays are often used as a biomonitoring test available to quantify and describe all of such a damage [2, 3].

According to the obtained results, the radon genotoxic effects on lymphocytes are increased at 22 °C. In general, the survival cells rate is reduced during ageing, as a result of damage to various cellular constituents by oxidative stress [23]. This stress must be increased in the presence of alpha particles. Damage may, however, also lead to the induction of apoptosis via the opening of a permeability transition pore (PTP) in the outer mitochondrial membrane and to leakage of cytochrome *c* and other proteins. Age related changes in the nuclear genome due to the damage, methylation or other processes, influence not only mitochondrial pathways, but also numerous pathways in the cytoplasm and other cellular compartments [23]. Repair of nuclear DNA via different enzyme activities (e.g. PARP [poly(ADP) ribosyl polymerase], or WRN, a helicase), ROS scavenging in the cytoplasm must be delayed when degradation of protein damages via proteasomes is increased. All these aspects have also an important impact on biological ageing [23].

From the database we know that additional features of replicative senescent cells involve resistance to apoptosis and altered differentiation, a large flat morphology and decreased saturation density in culture [20]. The local tissue microenvironment should be very important for the cell ageing. When the senescent human fibroblasts have been studied, it was observed that their metabolical state remains active for a long time by influencing the local tissue microenvironment [7]. At the same time, certain types of nuclear DNA damages, including oxidative damage and double strand breaks resulting from an altered balance between intensity of stress and efficiency of antioxidant cellular systems,

may eventually lead to the acquisition of additional senescent features, especially important for *in vitro* ageing [20]. Mocali and her collaborators have observed the differences in nuclear size among three cells populations of fibroblast cultures studied: noncycling young, presenescent, and senescent MRC5. She has observed a shift of large senescent nuclei distribution toward higher classes of damage, with ageing. These results are in accord with our observation, when it was observed the shift to an increased class of cells damage (Fig. 4).

Alterations in chromatin condensation status have been observed [18, 25], according with changes in fluorescence intensity and distribution observed in senescent nuclei. As we know, ionizing radiation deposits energy in the nucleus of the cell. DNA damage is induced and cellular responses to that damage affect the fate of the irradiated cell. DNA damage might activate cell cycle checkpoint control and cause the damaged cell to go into a protracted senescent state. Alternatively, if the damage is substantial, cell death may occur via a number of cellular pathways. We expect that the increase in radiation doses to increase the cells ageing and the DNA damage, but from Figure 7 a discrepancy has been registered, in the first 48 hours. A similar discrepancy for the ageing process has been signaled also by Mocali [20]. She has observed lower DNA damages in small senescent versus small young nuclei and the explanation was like a significant decrease in the amount of oxidized bases of senescent small nuclei representing a subset of cells that, although very limited in number, were qualitatively important because of their distinctive resistance to oxidative damage [20].

Cell turgidity takes place until it reaches a maximum volume, and is followed by membranes breaking as appropriate at a certain time with a decrease in the number of cells. Possibly the second stage depends also on the cell age, according to their lifetime in the blood. Young cells, for example, may be more resistant to breakage compared to old ones. Given the unfavorable environmental conditions in which cells are found after 24, 48 and more than that after 72 hours, it is still possible that cell death occurs faster than the first half of the Gaussian curve, which could characterize the entire process of ageing, and that could be highlighted while watching the viability of cells. If already the cell fragmentation took place, at migration, small resulting fragments cannot be quantified like comets, which can be another reason for error in assessing the degree of degradation.

The study of cell viability should be very important in assessing an implicate apoptosis, in ionizing radiation. As we know, the classical signs of apoptosis are cell shrinkage, increased cellular granularity, the formation of apoptotic bodies, and dilated nuclear membranes. In the study of Seddy Ibrahim [1], the nucleus of apoptotic cells HeLa shows condensation of chromatin after treatment with ZER when studies were done using transmission electron microscopy (TEM). The author has considered very important the study of the difference between the apoptosis and necrosis which imply many ultrastructural changes, in relation with cell cycle arrest and also the cell splitting in a number of apoptotic bodies

represents generally the final stage of apoptosis [1]. According to their results the micronuclei induction (MN) in radiation should be another explanation for our observed discrepancy between lesion score, cells number during ageing and DNA damage. For example, when human glioblastoma T98 G cell nuclei were individually irradiated with an exact number of helium ions, it was found that, when only one cell in a population of approximately 1,200 cells was target, cellular damage measured as induced micronuclei was increased by 20 %, assuming a bystander effect [19].

The induction of micronucleus has been also signaled with increasing culture time [21]. This appearance effect is not clear, but it could reflect differential growth rate of subpopulations of lymphocytes with different baseline MN frequencies, prolonged cell cycle of damaged cells, deteriorating culture conditions or higher micronucleation at longer culture times [14]. In genotoxicity tests it is crucial to understand whether an MN inducer acts via a clastogenic or an aneuploidogenic (or both) mechanism. The mode of action will influence the interpretation of the positive test result and the use of the data in risk assessment [5].

CONCLUSIONS

The number of lymphocyte damages in radon atmosphere, during the ageing process, is significantly increased. A linear dependence between the induced DNA lesions number in the first 48 hours has been observed by score lesion and tail factor, using the Colin's formula. Until around 48 ageing hours, the induced lesions number has been apparently increased for the lymphocytes culture kept in natural conditions. After 48 hours, equivalent with 162.6 kBq/m^3 radon concentration, should be possible to observe the increased number of induced lesions for the irradiated sample. The events which are happening after about 48 hours can improve the explanation of the mechanism implied in cells ageing and later its death, so to facilitate a good interpretation of the dose-effect relationship. On this model, the appreciation of dose-effect, dose-response must be improved by cells viability studies and cells counting.

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