Radiat Environ Biophys
DOI 10.1007/s00411-015-0587-4

ORIGINAL PAPER

Low-radiation environment affects the development of protection mechanisms in V79 cells

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Received: 8 April 2014 / Accepted: 16 January 2015

Abstract Very little is known about the influence of environmental radiation on living matter. In principle, important information can be acquired by analysing possible differences between parallel biological systems, one in a reference-radiation environment (RRE) and the other in a low-radiation environment (LRE). We took advantage of the unique opportunity represented by the cell culture facilities at the Gran Sasso National Laboratories of the Istituto Nazionale di Fisica Nucleare, where environment dose rate reduction factors in the underground (LRE), with respect to the external laboratory (RRE), are as follows: 10^3 for neutrons, 10^7 for directly ionizing cosmic rays and 10 for total γ-rays. Chinese hamster V79 cells were cultured for 10 months in both RRE and LRE. At the end of this period, all the cultures were kept in RRE for another 6 months. Changes in the activities of antioxidant enzymes (superoxide dismutase, SOD; catalase, CAT; glutathione peroxidase, GPX) and spontaneous mutation frequency at the hypoxanthine–guanine phosphoribosyl transferase (*hprt)* locus were investigated. The results obtained suggest that environmental radiation might act as a trigger of defence mechanisms in V79 cells, specifically those in reference conditions, showing a higher degree of defence against endogenous damage as compared to cells grown in a very low-radiation environment. Our findings corroborate the hypothesis that environmental radiation contributes to the development of defence mechanisms in today living organisms/systems.

Keywords Radiation environment/background · V79 cells · Spontaneous mutation · Antioxidant enzymatic activities · Adaptive response · Defence mechanisms

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Introduction

Life evolved over billions of years in environmental conditions that include the exposure to radiation from both space and the Earth mantle. Many studies have been devoted to the influence of factors such as temperature, pressure and atmospheric composition on life biochemistry (Banister et al. 1973; Ducommun et al. 2000; Havlicek and Slama 2011; Sandabe and Chaudary 2000; Tagaki et al. 1995). In contrast, fewer works have assessed the influence of radiation environments on cell metabolism. The pioneering works of Planel et al. (1976, 1987) show that the growth of protozoan and cyanobacterium cells was inhibited when they were cultured in a low-radiation environmental laboratory in the Pyrenees Mountains. More recently, Smith et al. (2011) observed a stress response in mammalian and bacterial cells grown under reduced radiation environmental conditions at the Waste Isolation Pilot Plant (WIPP), USA.

The underground Gran Sasso National Laboratory (LNGS) of the Italian Institute of Nuclear Physics (INFN), thanks to its very low-radiation environmental conditions,

Published online: 31 January 2015

was constructed to boost underground physics and, more generally, to study quite rare events such as proton decay or solar neutrino detection. In this underground laboratory, shielded by at least 1,400 m of rock overburden, cosmic radiation is almost absent (The MACRO Collaboration 1990), and, given the scarce uranium and thorium content in the dolomite rocks of the mountain, the neutron flux is reduced by a factor of 10^3 with respect to external values (Rindi et al. 1988; Belli et al. 1989). In addition, being the mountain of sedimentary origin, the natural occurrence of γ and μ radiation is minimal and further reduced by the low-activity concrete lining of the laboratory walls.

Radon concentration is kept at a very low level by an efficient ventilation system that pumps low-radon-concentration air from the outside into the laboratory. Thanks to these features, underground LNGS represents a unique opportunity to explore an environment where the presence of radiation is strongly reduced.

We took advantage of this opportunity at LNGS and set up an underground cell culture laboratory to investigate possible differences in cell metabolism between parallel biological systems, one kept in a "reference"-radiation environment (RRE) and the other in a low-radiation environment (LRE).

A first study using the yeast strain *Saccharomyces cerevisiae* showed that cells grown in LRE for 1 week (about 120 generations) exhibited reduced repair efficiency when exposed to the genotoxic agent methyl methanesulphonate (MMS), as compared to cells grown in RRE at the University of Rome (Satta et al. 1995).

The study was then extended to human and rodent mammalian cells. Parallel cultures of Chinese hamster V79 cells were maintained for up to 9 months in exponential growth in the two radiation environmental conditions (LRE at the underground Gran Sasso laboratory and RRE at the Istituto Superiore di Sanità in Rome). Upon evaluating the basal activity of several antioxidant enzymes and of the spontaneous and radiation-induced mutation frequency (MF), differences were found between the two cultures (Satta et al. 2002).

Using the same experimental approach, a lymphoblastoid cell line (TK6) of human origin was cultured for up to 6 months at the above LRE and RRE laboratories. The cells kept at LRE were less efficient at scavenging endogenous reactive oxygen species (ROS) and more sensitive—in terms of induced micronuclei—to acute exposure to radiation (Carbone et al. 2009, 2010).

Although the reported experiments revealed differences between RRE- and LRE-cultured cells, we may not exclude that, after so many generations (e.g. 9 months roughly corresponding to 540 V79 cell duplications), mutant clones with different characteristics had been selected by chance in the cultures kept in different radiation environments.

Here, we further investigated the repair efficiency of V79 Chinese hamster cells in different radiation environments by analysing the activities of relevant antioxidant enzymes, together with their mRNA levels, and their spontaneous mutation frequency. This time, as RRE laboratory we used the unshielded cell culture laboratory set-up outside the tunnel at the LNGS. To reduce the probability that random mutant selection could affect the results, sister cultures were grown in RRE and LRE. As a novel approach, we tested whether the changes acquired during 10 months of culture in LRE reverted after further 6 months in RRE.

Materials and methods

Dosimetry

Radiation environment dosimetry was obtained by measurement and evaluation of γ-rays of any origin, cosmic rays (neutrons and directly ionizing particles), α-particles from ^{222}Rn and its daughters. The internal dose due to the auto-irradiation of cells from ^{40}K was evaluated as well.

The ^{222}Rn dose component was determined by measuring ^{222}Rn concentration in air with a radon meter (alphaGUARD, Genitron Instruments GmbH) and then converting it into radiation dose to cultured cells using a model devised by Jostes et al. (1991) for in vitro cultures of Chinese hamster ovary cells exposed to high activities of ^{222}Rn. The model was applied here to Chinese hamster V79 lung fibroblasts at environmental ^{222}Rn activity levels. To convert ^{222}Rn environmental activity to dose to the cells, daughter products of ^{222}Rn decay were assumed to be in secular equilibrium (^{222}Rn:^{218}Po:^{214}Po). We also assumed that ^{222}Rn concentrations in air and liquid phase—i.e. in the culture medium—were in equilibrium and that the liquid phase/air ratio of ^{222}Rn activity was 0.167 at 37 °C.

To prevent the accumulation of ^{222}Rn activity indoors, the underground LNGS laboratories are equipped with a powerful air ventilation system that collects air from outside the underground laboratory and expels it outdoors.

The dose rate due to neutrons at RRE was assessed on the basis of the direct measurements of the atmospheric neutron fluxes at LNGS external site done by Rindi et al. (1988) in the 0.025 eV–10 MeV energy range and by Bonardi et al. (2010) and Olsher et al. (2010) in the 10–500 MeV energy range. By combining these data, fluxes from 0.025 eV to 500 MeV were obtained in six energy regions. To convert the neutron flux to absorbed dose rate, we applied to each energy region the Kerma factor for water listed in ICRU Report 46 (1992) and summed up the resulting values.

The neutron dose rate at LRE was derived from that at RRE, applying the reduction factor of about 0.7×10^3 found by Rindi et al. (1988) for the thermal neutron flux measured underground with respect to the external LNGS site, and considering that the same factor also holds for epithermal and fast neutrons. The underground neutron flux measurement was reviewed by Wulandari et al. (2004).

Terrestrial and cosmic γ-ray dose was measured with thermoluminescence dosimeters, namely TLD 700H detectors. They were placed in plastic cell culture flasks and kept in the incubator. A 5-cm-thick iron shielding, which reduces the measured γ-ray dose by a factor of about 10 for the γ-ray spectrum of interest, was built around the cell culture incubator.

The internal dose due to γ and β ^{40}K decay was measured with a Ge spectrometer (Hewlett-Packard) on a pellet of 2×10^9 cells collected over a period of 1 month.

Culture conditions

Chinese hamster V79 lung fibroblasts were grown as monolayer in Eagle's minimal essential medium supplemented with 10 % foetal calf serum (FCS), 1 mmol dm^{-3} glutamine, 50 U dm^{-3} penicillin and streptomycin. All reagents used throughout the entire experiment were from GIBCO, presently Invitrogen, and from the same batches.

Starting from the same frozen sample, duplicate and independent V79 sister cultures were settled at RRE (cultures A and B) and at LRE (cultures C and D) laboratories. Duplication was aimed at minimizing the effect of possible random selection of fast-growing mutants that could outnumber wild-type cells in the overall culture. Indeed, assuming that the spontaneous mutation frequency in V79 cells is, typically, of the order of 10^{-4}–10^{-5} for a given genetic locus and that one mutated locus is sufficient to determine the observed features, the probability of obtaining by chance the same mutant in two independent sister cultures (A and B, or C and D) is of the order of 10^{-8}–10^{-10}, or less. In the attempt to further reduce this possible effect, each culture was divided into four subcultures which, however, were pooled together before the various assays.

Passages of cells were carried out three times a week in order to maintain them in the exponential phase of growth. After 10 months, LRE-grown cultures C and D were brought to the RRE laboratory and cultured for another 6 months together with RRE-grown cultures A and B.

The assays for the end points considered were performed at the beginning of the experiment (t_0), after 10 months ($t_0 + 10$) and after 16 months ($t_0 + 16$) of culture. All the cultures were periodically frozen for backup and/or further analysis.

Cell extracts preparation and enzymatic activity assays

Enzymatic activity assays were made on cell extracts obtained from the pool of the four subcultures of culture A; the same applied to cultures B, C and D. Cells were suspended at the concentration of 10^7 cells/mL in 10 mM phosphate buffer, pH 7.0, containing 10 mM dithiothreitol (DTT) for glutathione peroxidase assay, or Triton X-100 for catalase and total SOD assays. All chemicals were purchased from Sigma unless otherwise specified. Cell suspensions were thawed and frozen three times in liquid N_2, homogenized and centrifuged at 1,000*g* for 30 min at 4 °C. The resulting cell extracts were used for the spectrophotometric measurement of enzymatic activity and protein content.

Total superoxide dismutase (SOD) activity in cell extracts was assayed by optical density measurements at 480 nm and 30 °C by its ability to inhibit the epinephrine autoxidation, according to Sun and Zigman (1978). The reaction was carried out in 50 mmol/L sodium carbonate buffer, pH 10.2, and was initiated by the addition of 0.1 mmol/L adrenaline. A standard curve, with a purified Cu–Zn bovine SOD, was obtained by plotting the inverse values of the amount of enzyme used versus the percentage of inhibition observed. This standard curve was used to determine the amount of extract necessary for a 50 % inhibition. One unit of SOD was defined as the amount of the enzyme required to halve epinephrine autoxidation.

Catalase (CAT) activity was measured at 240 nm and 25 °C by following the rate of reduction of hydrogen peroxide, according to Aebi (1970). The reaction mixture contained 100 mmol/L potassium phosphate buffer pH 6.8 and 10 mmol/L H_2O_2. One unit of CAT is defined as the amount of the enzyme required to reduce 1 μmol of H_2O_2 per min.

Selenium-dependent glutathione peroxidase (GPX) activity was assayed according to Paglia and Valentine (1967). The assay solution contained 50 mM monobasic potassium phosphate, pH 7.0, 1 mM EDTA, 1.5 mM sodium azide, 0.4 U glutathione reductase, 0.45 mM GSH, 0.2 mM NADPH and 0.25 mM H_2O_2 as substrate. The oxidation of NADPH was followed at 340 nm and 25 °C. One unit of SeGPX was defined as the amount of enzyme required to oxidize 1 μmol of NADPH per min.

Protein concentration was determined by Protein Assay Kit (Bio-Rad) as described in Bradford (1976) using bovine serum albumin (BSA) as standard.

Gene expression

RNA extraction and quality analysis

Total RNA was isolated using TRIzol® RNA Isolation Reagents (Life Technologies, Carlsbad, California) from thawed cell culture from each experimental point. RNA concentration and purity were determined by measuring the absorbance at 260 and 280 nm with a Lambda 2 Spectrophotometer (PerkinElmer, Waltham, Massachusetts); 1 mg of total RNA was run on a 1 % denaturing agarose gel to verify RNA integrity.

Real-time qPCR (RT-qPCR)

One milligram of total RNA from each individual sample was retrotranscribed into cDNA by GeneAmp RNA PCR Kit (Life Technologies, Carlsbad, California), following the manufacturer's instructions. qPCR was performed using Premix Ex Taq™ (Probe qPCR) ROX™ plus (TaKaRa Bio Inc., Otsu, Shiga, Japan), according to the manufacturer's instructions. Amplification included the following Chinese hamster genes: rodent glyceraldehyde phosphate dehydrogenase (*Gapdh*) (4308313, VIC™ Probe), glutathione peroxidase 1 (*Gpx1*) (ACCCCACTGCGCTCATGACCGA, FAM/MGB Probe), glutathione peroxidase 2 (*Gpx2*) (CCTGGTAGTTCTCGGCTTCCCTTGCA, FAM/MGB Probe), glutathione peroxidase 3 (*Gpx3*) (CACCGGACCACAGTCAGCAACGTC, FAM/MGB Probe) and glutathione peroxidase 4 (*Gpx4*) (TGGTTTACGAATCCTGGCCTTCCCCT, FAM/MGB Probe) (all from Life Technologies, Carlsbad, California). For the following Chinese hamster genes, RT-qPCR was performed by RealMasterMix SYBR Rox 2,5 (Eppendorf, Hamburg, Germany) according to the manufacturer's instructions: *Cat*, *Sod*, selenium-binding protein 1 (*Sbp1*) and *Gapdh*. Specific primer pairs were designed by Primer Express Software (ABI); their sequences are listed in Table 1. All reactions were performed in duplicate in the ABI 7000 Real-Time PCR System (Life Technologies, Carlsbad, California), and relative quantification was done with the ΔΔCT method (Kenneth and Schmittgen 2001) provided by ABI, using the abundance of *Gapdh* mRNA as endogenous housekeeping control.

Spontaneous mutation frequency

Mutation experiments were carried out on cells obtained from the pool of the four subcultures of culture A, as well as those of cultures B, C and D. The hypoxanthine–guanine phosphoribosyl transferase (*hprt*) locus was evaluated twice with a 48-h interval. Petri dishes of 90 mm diameter (ten for each culture) were seeded with 3×10^5 cells/dish in the presence of 6-thioguanine (0.5 μg/ml) (Sigma) and 5 % FCS. At the same time, 200 cells/dish were plated with complete medium in each of four Petri dishes (60 mm diameter) to determine the cloning efficiency.

Mutation experiments were performed at times t_0, $t_0 + 10$ and $t_0 + 16$ on cultures A, B, C and D. The same measurements were repeated once on cells from frozen samples, thawed some months later. For each culture, the evaluated MF was the mean of four measurements, two on continuous growing cells and two on frozen samples.

Table 1 Primer sequences

Primer	Sequence 5′–3′
GPx1 sense	CTCACCCGCTCTTTACCTTCCT
GPx1 antisense	ACACCGGAGACCAAATGATGTACT
GPx2 sense	GTCGCATCACTCTGAGGAACA
GPx2 antisense	CAGTTCTCCTGATGTCCAAATTG
GPx3 sense	CAAACCTGTTATGCGCTGGTA
GPx3 antisense	CCTGCCGCCTCATGTAGGAC
GPx4 sense	TGAGGCAAAACCGACGTAAACTACA
GPx4 antisense	GTTCCTGCTTCCCGAACTG
CAT sense	GAATGGCTACGGCTCACACA
CAT antisense	CAAGTTTTTGATGCCCTGGTCG
SOD sense	GGTGGTCCATGAGAAGCAAG
SOD antisense	CGATCACACCACAAGCCAAG
SBP1 sense	TCCCGCCTCTATGTGGTGGA
SBP1 antisense	AGTGGCTGGTGTGCAGATTG

Data analysis and statistical tests

Statistical differences between the results obtained from different cultures for the various end points were analysed using the Student's *t* test.

Results

Dosimetric characterization of the radiation environment

Among the various components of environmental radiation, one relevant contribution to the overall dose to the cells comes from radon decay products. Actually, the underground ^{222}Rn concentration can reach values of the order of 100 Bq/m^3. In the presence of a ventilation system, however, the concentration drastically diminishes to 5 Bq/m^3, which is the same value obtained in the RRE laboratory. The corresponding dose rate was calculated to be 1.7 nGy/h in both LRE and RRE (see "Materials and methods").

The internal dose due to γ and β ^{40}K decay has also been taken into account. As measurements of ^{40}K decay did not exhibit a significant signal above the background, this contribution was evaluated by equating the ^{40}K concentration in cells to that of the human body (UNSCEAR 2000).

The dose rate related to the γ-rays component was measured by TLD dosimeters. The values obtained were 3.6 and 34 nGy/h at LRE and RRE, respectively.

The dose rate due to directly ionizing component of cosmic rays (mainly muons) was assumed to be negligible for the underground laboratory (LRE), and of about 39 nGy/h at RRE, as evaluated on the basis of the UNSCEAR 2000 data (Sources, Annex E).

The neutron dose rate calculated at RRE as described in Materials and Methods was 2.5 nGy/h. A similar calculation, using the dose equivalent coefficients (ICRU Report 57 1998; Olsher et al. 2010) instead of Kerma coefficients, yielded an equivalent dose rate value of 20 nSv/h, which—even though not specific for cells—can be used for comparison with the current UNSCEAR estimate (2000, 2008). At sea level, this parameter is, on average, ∼80 μSv/year (UNSCEAR 2000, 2008), i.e. ∼9 nSv/h. After multiplying by a factor of 2.5 for 1,000 m altitude, an average value of ∼22 nSv/h is obtained, which compares well with the present calculation based on specific measurements.

The neutron dose rate at LRE, taking into account the reduction factor of nearly 10^3, can be considered negligible as compared to that at RRE.

Table 2 summarizes the dosimetric measurements and estimations for both laboratories.

The total dose rate reduction for the low-LET (linear energy transfer) component at LRE was found to be about fourfold lower than at RRE. A higher reduction (about 14-fold) was calculated for the high-LET component, which is known to have higher biological effectiveness than low-LET radiation. It should be noted that ^{40}K and radon contributions are the same in both contexts.

Antioxidant enzymatic activity

The biochemical activities of enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX), all of them involved in quenching the damage produced by reactive oxygen species (ROS), were assayed in V79 cells at the beginning of the culture (t_0) and on cultures A, B, C and D after $t_0 + 10$ and $t_0 + 16$ months of culture (Fig. 1).

The biochemical activities measured in A and B (RRE) cultures were not statistically different ($p > 0.05$). Similar results were obtained for the C and D (LRE) cultures. Therefore, data are presented as average values for each pair of cultures, according to the radiation environmental condition.

Figure 1a, b shows the enzymatic activities of SOD and CAT, respectively. The values for SOD and CAT tend to decrease with culture time, with little differences between RRE and LRE.

The results for GPX activity (Fig. 1c) are, instead, very different: GPX activity of RRE cultures was twice as high as at t_0, while that of LRE cultures was about two times lower, showing a ratio of about 4 between the two environmental conditions.

Table 2 Evaluation of dose rate for the main components of the radiation environment

Source	Dose rate at RRE (nGy/h)	Dose rate at LRE (nGy/h)
Directly ionizing cosmic rays (low LET)	39[a]	Negligible[c]
Neutrons from cosmic rays (high LET)	2.5[b]	Negligible[c]
Total γ-rays (cosmic and terrestrial, low LET)	34[d]	3.6[d]
^{222}Rn and daughters (high LET)	0.17[e]	0.17[e]
^{40}K (internal exposure, low LET)	19[f]	19[f]
Total (rounded)	94.7	22.8
Low LET (rounded)	92.0	22.6
High LET (rounded)	2.7	0.2

[a] Evaluation based on UNSCEAR 2000 and 2008

[b] Evaluation based on measures by Rindi et al. (1988), Bonardi et al. (2010) and Olsher et al. (2010) applying the Kerma factors for water listed in ICRU Report 46 (1992)

[c] As above, applying the experimental reduction factors of the rock coverage

[d] TLD measurements

[e] Calculation based on the application of the model by Jostes et al. (1991) to the measured Rn concentration

[f] Evaluated by equating ^{40}K concentration in cells to that in the human body and applying the data from UNSCEAR 2000

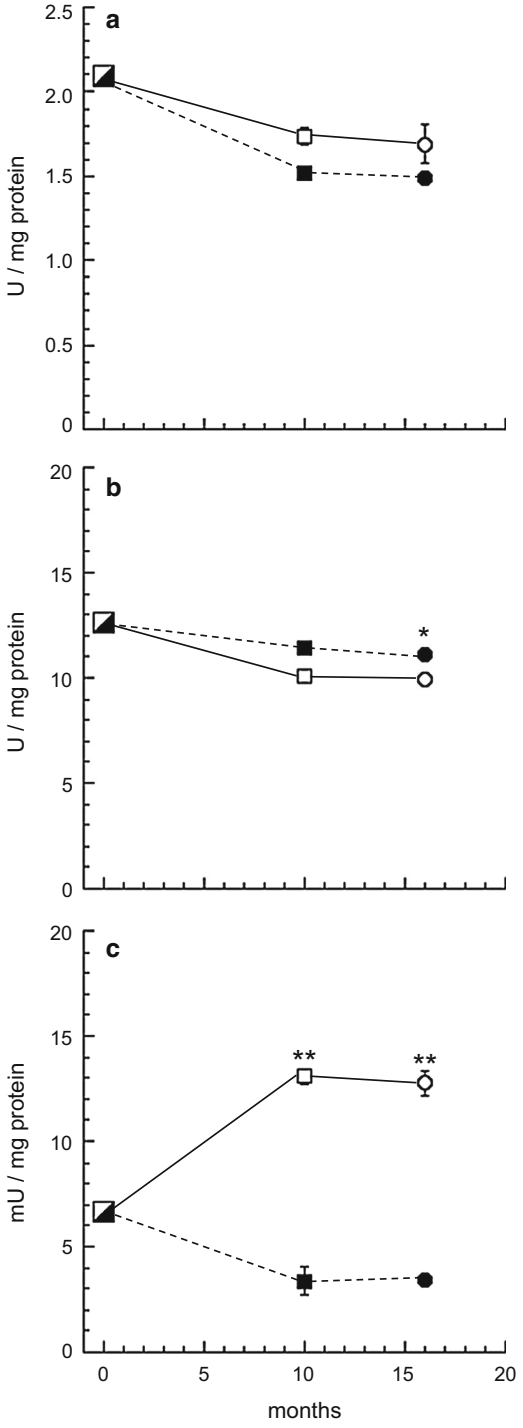

Fig. 1 Antioxidant enzymatic specific activities in V79 cells at 0 *(half-filled square)*, after 10 months of culture at RRE (*open square*: A and B) or LRE (*closed square*: C and D) and after further 6 months of all the cultures at RRE (*open* and *closed circles*). Data points represent the mean value of two independent determinations obtained from sister cultures (A and B or C and D) pooled together; *error bars* are the corresponding semi-dispersions, in some cases they are smaller than the *symbols*. Statistical significance of the differences, according to the Student's *t* test, is also shown (* $p < 0.05$ and ** $p < 0.01$). **a**: SOD; **b**: CAT; **c**: GPX

The additional 6 months of growth at RRE of all the cultures (previously maintained at RRE or at LRE) did not significantly change SOD, CAT or GPX enzymatic activities (Fig. 1). None of the measured activities reverted to its original level. This fact is particularly interesting for GPX activity, which shows highly significant differences between RRE and LRE (Student's *t* test, $p < 0.01$), suggestive of a permanently acquired feature.

Gene expression

To evaluate whether enzymatic activities are regulated at gene expression level, a real-time quantitative PCR analysis was performed on thawed V79 cells that had been frozen at the beginning of the culture (t_0) and after $t_0 + 10$ and t_0 - + 16 months of culture. Similar to the enzymatic activities, the results from cultures A and B, and from cultures C and D, were not statistically different, so that data are presented pooling together the values obtained from sister cultures.

Figure 2 shows the PCR analysis of different *Gpx* isoforms, namely *Gpx1*, *Gpx2*, *Gpx3*, and *Gpx4*. Although mRNA production for Gpx isoforms showed high variability among samples, Student's *t* test showed that after $t_0 + 16$ months of culture, the difference in Gpx2 values is statistically significant ($p < 0.01$) between cells always grown at RRE and cells kept at LRE for the first 10 months and at RRE for the further 6 months. Similarly, a statistically significant difference ($p < 0.01$) was observed for Gpx4 values: Gpx2 showed a 1.5-fold reduction, while Gpx4 a 2.2-fold increase in RRE cultured cells with respect to LRE-cultured ones. All the other Gpx isoforms showed no difference between RRE and LRE.

Sod mRNA production (Fig. 3a) is scarcely affected by radiation environmental conditions or culture time. There was about twofold increase in *Cat* mRNA production (Fig. 3b) after 10 months in RRE cultured cells with respect to the LRE culture even though this difference was not statistically significant owing to the high standard deviation for the RRE value. After the additional 6 months of growth at RRE, mRNA production increased more for LRE than for RRE culture, and the ratio between them showed about a 0.5-fold decrease.

The product of Sbp1 gene is a selenium-binding protein (SBP1) involved in the regulation of Se-GPX (GPX1-4) enzymatic activity. It acts by subtracting selenium, essential co-factor for glutathione peroxidase enzymes: a decrease in SBP1 induces an increase in total cell GPX activity. *Sbp1* mRNA (Fig. 3c) does not change with respect to t_0 under either environmental radiation condition after 10 months of culture, while it decreases after further 6 months at RRE, independently of the previous environmental radiation condition.

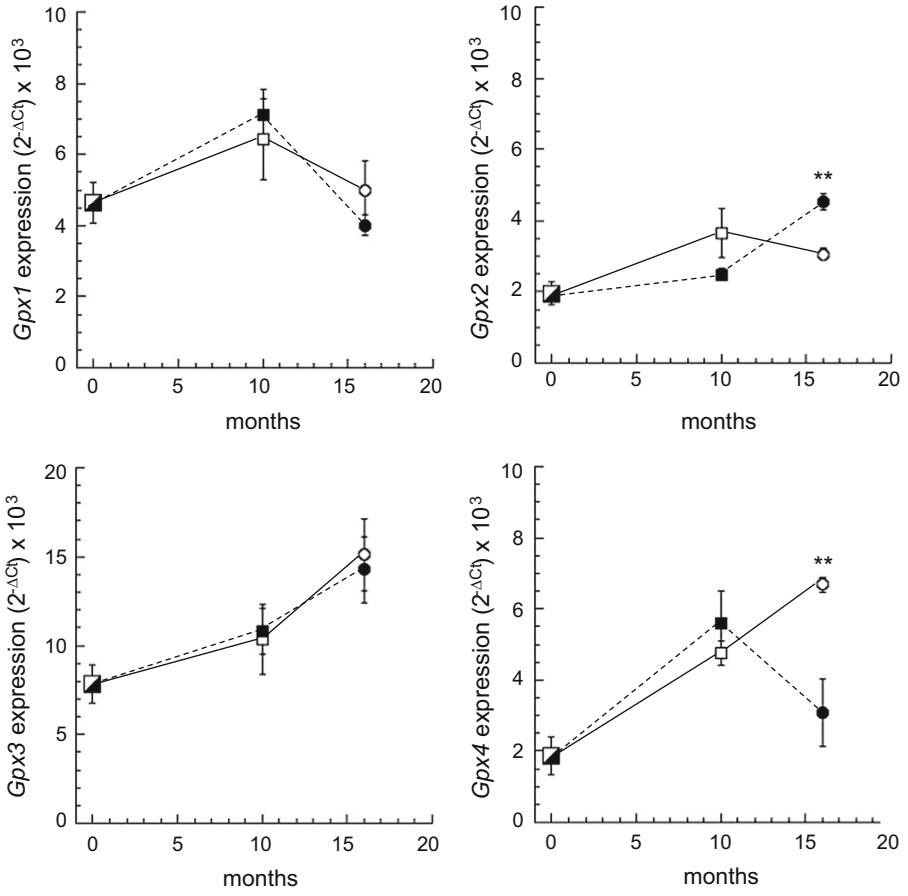

Fig. 2 Real-time quantitative PCR analysis of the four *Gpx* isoforms: *Gpx1*, *Gpx2*, *Gpx3* and *Gpx4*, at 0 (*half-filled square*), after 10 months of culture at RRE (*open square*: A and B) or LRE (*closed square*: C and D) and after further 6 months of all the cultures at RRE (*open* and *closed circles*). Data points represent the mean value from sister cultures (**a** and **b** or **c** and **d**) for a total of four independent determinations with their standard errors; in some cases, the *error bars* are smaller than the *symbols*. Statistical significance of the differences, according to the Student's *t* test, is also shown (** $p < 0.01$)

Spontaneous mutation frequency (MF)

Likewise, spontaneous MF at the *hprt* locus was assayed at the beginning of the cultures (t_0) and on cultures A, B, C and D after $t_0 + 10$ and $t_0 + 16$ months of culture. The results obtained from pooling together data from sister cultures A and B and from sister cultures C and D are shown in Fig. 4.

After 10 months at LRE, sister cultures exhibited a more than twofold increase in the basal level of MF with respect to t_0. Such an increase, however, is within the test variability in this cell line (Belli et al. 1991, 1992; Sapora et al. 1991; Thacker et al. 1979; Stoll et al. 1995; Bacsi et al. 2006). Conversely, the MF of the RRE cultures did not significantly change, whereas a clear difference is evident after the additional 6 months of permanence for all the cell cultures (A, B, C and D at RRE). At this stage, with respect to t_0, the MF of the cultures previously kept for 10 months at RRE increased by a factor of about 2, while that of cultures kept for 10 months at LRE increased by a factor of about 7.

Discussion

In this paper, we further investigated the influence of environmental radiation in modulating defence mechanisms in V79 Chinese hamster lung fibroblasts. We carried out the analysis on sister cultures kept for 10 months at LLR and also investigated whether the observed biological responses would revert if the cells were brought back to the RRE.

As in our previous paper (Satta et al. 2002), we focused on the activities of antioxidant enzymes and on mutation frequency. It should be noted, though, that in the mentioned study the LRE was the same as for the present experiments, while the RRE was located at the Istituto Superiore di Sanità in Rome, not at the external LNGS laboratory. As a consequence in the present set-up, the difference in dose rate between RRE and LRE was lower than in the previous study. On the other hand, performing all the experiments at the LNGS presented the advantage of reducing the experimental variability since the same operators were in charge of the cell culture procedures in

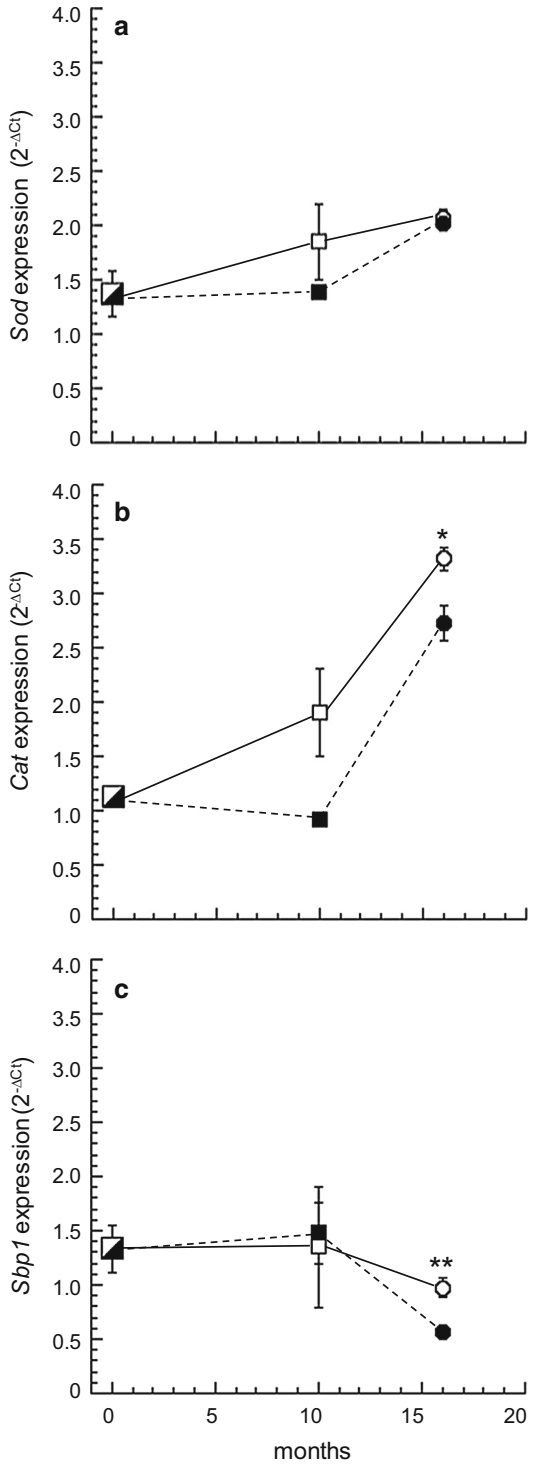

Fig. 3 Real-time quantitative PCR analysis of *Sod*, *Cat* and *Sbp1* at 0 (*half-filled square*), after 10 months of culture at RRE (*open square*: A and B) or LRE (*closed square*: C and D) and after further 6 months of all the cultures at RRE (*open* and *closed circles*). Data points represent the mean value from sister cultures (**a** and **b** or **c** and **d**) for a total of four independent determinations with their standard errors; in some cases, the *error bars* are smaller than the *symbols*. Statistical significance of the differences, according to the Student's *t* test, is also shown (* $p < 0.05$ and ** $p < 0.01$)

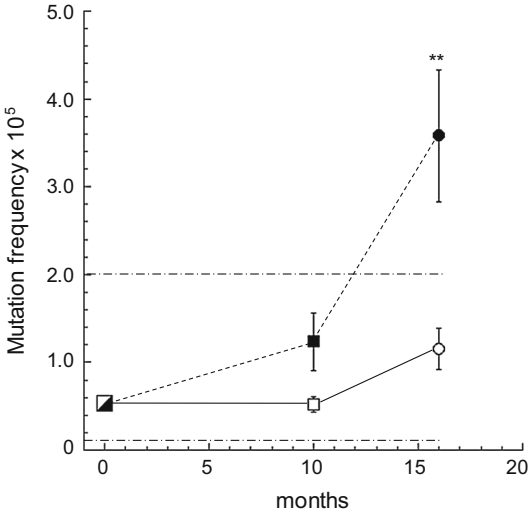

Fig. 4 Spontaneous *hprt* mutation frequency in V79 cells at 0 (*half-filled square*), after 10 months of culture at RRE (*open square*: A and B) or LRE (*closed square*: C and D) and after further 6 months of all the cultures at RRE (*open and closed circles*). Each data point represents the mean value of four measurements obtained from two independent determinations on each of the two sister cultures (**a** and **b** or **c** and **d**); the *error bars* are the corresponding standard errors, in some cases they are smaller than the *symbols*. Statistical significance of the differences, according to the Student's *t*-test, is also shown (** $p < 0.01$). The *dashed lines* represent the range of the measured data reported in the literature (Belli et al. 1991, 1992; Sapora et al. 1991; Thacker et al. 1979; Stoll et al. 1995)

both RRE and LRE laboratories. This is an aspect that, together with the implementation of replicate cultures, reduces the impact of possible confounding factors.

Among the antioxidant activities of CAT, SOD and GPX enzymes, we found that only GPX activity significantly decreased at LRE and remained so even after further 6 months at RRE. In the attempt to gain more insight into this phenomenon, we investigated whether GPX activity is regulated at gene expression level. The mRNA measurements of CAT, SOD and four major isoforms of GPX enzymes did not show variations between LRE and RRE that can explain our findings. The major differences observed in Gpx2 and Gpx4 expression levels do not significantly affect the total GPX activity: the results are divergent and these isoforms represent only a minor component of the total Gpx content of the cell.

We also measured *Sbp1* mRNA because the derived protein is able to rip off the selenium from the GPX active site and thus inhibit GPX activity. The results clearly showed that SBP1 was not involved in this regulation. Therefore, at present we are unable to formulate a hypothesis on how and why GPX activity is affected by LRE.

Indeed, in the present study, there is a discrepancy with our previous GPX results (Satta et al. 2002) where, after

9 months of culture, GPX activity at LRE was higher than at RRE. Although at the moment we cannot explain such discrepancy, the present findings at RRE are consistent with literature data (Kang et al. 2005; Lee et al. 2003).

The mutation data, instead, are in agreement with the results of our previous experiment (Satta et al. 2002). As in the past, we found an increase in spontaneous MF after 10 months of cell culture at LRE; moreover, when these cells were kept for further 6 months at RRE, the MF increased to an extent comparable to the increase observed in the same cell line after a dose of 2.5 Gy X-rays (Belli et al. 1991).

These findings indicate that cells accumulate genetic errors with culture time, that are dependent on the radiation environment. The persistence of high *hprt* MF and low GPX enzymatic activity after the LRE cultures are moved to RRE for 6 months might indicate that the permanence at LRE inhibits the triggering of cellular defence mechanisms. In other words, keeping cells in LRE would make them go into a state in which the mechanisms involved in cellular DNA damage response pathways run less efficiently than in RRE. When these cells are moved to RRE, they keep memory of this state for at least another 6 months.

Then, it is reasonable to speculate that the responses observed in our four experiments, i.e. the present one and the three already published on yeasts and rodent/human mammalian cells, can be related to the difference in spectrum and dose rate of the two radiation environments, RRE and LRE.

Inside the Gran Sasso mountain, the radiation environment is composed essentially of low-energy γ-rays of local origin (low-LET radiation) whose spectrum extends to about 3 MeV. The thickness and sedimentary nature of the overburden almost eliminate the contribution of cosmic rays and neutrons (Rindi et al. 1988).

The possibility that the observed effects were due to $^{\cdot}$OH free radicals seems to be ruled out by the calculation made by Pollycone and Feinendegen (2003) where the rate of DSB-type lesions endogenously generated by DNA oxy-adducts outnumbers the DSB rate caused by environmental radiation by a factor up to 10^3. Accordingly, exposure to this radiation should have a negligible biological effect and "a fortiori" should have negligible consequences on the differences between RRE and LRE.

However, in recent years, a great deal of evidence has been accumulated on the effects of exposure to low levels of ionizing radiation (Morgan and Bair 2013; UNSCEAR 2010). To put into a right perspective the comparison between endogenous and radiation-induced damage, and their respective roles, it is necessary to consider a number of features besides the amount of radiation-induced $^{\cdot}$OH, on which the above argument is based. First of all, their spatial distribution is central: the endogenous production of $^{\cdot}$OH is mainly confined in organelles outside the nucleus (see, e.g., the review by Rahal et al. 2014) so they may have little impact on nuclear DNA, as compared to radiation-induced $^{\cdot}$OH, which has no preferential localization at this level. Secondly, both the indirect action (mainly $^{\cdot}$OH from radiolysis) and the direct action of the radiation (direct DNA ionization) are to be taken into account. It is well known that the severity of DNA damage is associated with the spatial correlation of the energy events deposited by the radiation. Clustered DNA damage is the most biologically relevant radiation-induced DNA damage (Ward 1985, 1994; Goodhead et al. 1993; Goodhead 1994; Nikjoo et al. 1997) as it is expected to be less readily repaired as compared to most cellular-induced damage. Since the likelihood of clustered DNA damage sites arising endogenously is low (Sutherland et al. 2003, Bennett et al. 2004), this damage is regarded as a signature of ionizing radiation (O'Neill and Wardman 2009).

Clustered ionizations are typically produced by high-LET radiation, via the significant fraction of the dose deposited by low-energy secondary electrons (Nikjoo and Goodhead 1991). Low-LET radiation can also produce them, though less effectively. For high-LET radiation, having an RBE (relative biological effectiveness) >1, the yield of OH radicals decreases when LET increases. This clearly indicates lack of correlation between $^{\cdot}$OH yield and the production of biologically relevant damage by ionizing radiation, consistently with the notion that, in addition to the indirect action, also the direct action—whose contribution depends on radiation quality—is to be accounted for.

The irradiation of cell populations in the present experiment is highly inhomogeneous: a small number of cells can receive a relatively high specific energy (this quantity is used instead of the dose for describing stochastic events in such microscopic masses). After 10 months, the total dose to cell populations is about 0.84 and 0.17 mGy at RRE and LRE, respectively. As most of it is delivered by low-LET radiation, and assuming that a dose of 1 mGy from γ-rays gives a mean of 1 track in a typical cell nucleus (Goodhead 1992), the average number of tracks per nucleus received from low-LET radiation by a cell or its progenitors after 10 months is roughly 0.84 at RRE and 0.17 at LRE. Moreover, due to the random nature of the energy deposition, each of these tracks will deliver a specific energy from 0.01 to 25 mGy (Goodhead 1992). Assuming a Poisson distribution, there is still a little but significant probability that a nucleus of a single cell or its progenitors has been traversed by more than one track at RRE, while this event is very unlikely at LRE. At RRE, there is also a significant contribution from high-LET radiation, the tracks of which, although much less frequent,

can deliver relatively high specific energy. For example, it was evaluated (Goodhead 1992) that 1 track of 1 MeV neutrons corresponds to an average specific energy to a typical cell nucleus of about 100 mGy.

In this regimen of very low doses and dose rates, the inhomogeneity of energy deposition among cells and within cells (at the micro- and nano-metric levels), characteristic and dependent on radiation quality, is a feature deserving particular attention (Tabocchini et al. 2012). Indeed, in these conditions, cell–cell communication can play an important role (e.g. Trosko et al. 2005). Even if few cells are directly affected by the radiation, cell–cell communication is able to propagate such perturbations to other (neighbouring) cells and amplify the number of perturbed cells (Campa et al. 2013), a phenomenon known as "bystander effect".

The heterogeneity of energy deposition in single cells seems an essential factor in this kind of response. For example, it has been reported that triggering the production of a bystander signal is an "all-or-none" response (Schettino et al. 2005). The energy deposited in a small target, typically a cell, seems to activate responses in a much larger target, and if the energy deposition in a cell is too low, these mechanisms are not activated (ICRU 2011). Low-dose thresholds for activating cooperative protective processes have been invoked to model nonlinear dose–response relationships (Scott 2006). Complex responses with a mitigation of effects at low doses, but not at ultra-low doses, are well known in cell systems. In particular, a transition from an enhanced radiosensitivity dose region to a region with induced radioresistance was observed for clonogenic survival in V79 cells (Marples and Joiner 1993) and for chromosomal changes in a mouse model (Hooker et al. 2004).

Taking into account the possible activation of physiological DNA damage control systems by the radiation-induced damage, low cell doses have been claimed to have a dual effect, one of which causes DNA damage and the other stimulates the physiological system that constantly controls the sources and consequences of the level of steady-state endogenous DNA damage (Pollycove and Feinendegen 2003; Feinendegen et al. 1995, 1996). As an effect of this "adaptive" protection, at low doses reduction of damage from endogenous sources may be equal to or outweigh radiogenic damage induction (Feinendegen 2005). In the context of the present experiment, it is tempting to speculate about the different effects of the radiation environment at RRE compared with LRE and their origin, in terms of cell response to the radiation-induced perturbations. The results hereby reported could be reasonably attributed to cell defence mechanisms that are triggered when a minimum number of cells receive a minimum amount of damage. This condition could hold at RRE, owing to the relatively higher low-LET dose rate and to the presence of a significant high-LET component, but not at LRE. According to this interpretation, the cultures kept at LRE are less capable to cope with the overall damage, as revealed by the high *hprt* MF and low GPX enzymatic activity, resulting from the loss of defence stimulation as a consequence of a reduced damage level. The persistence of high *hprt* MF and low GPX enzymatic activity after the LRE cultures are kept for 6 months at RRE might indicate that a long time is needed to regain the lost level of defence systems. This may be consistent with the hypothesis that the defence stimulation at RRE is acquired by epigenetic regulation. Indeed, some findings suggest that low doses of ionizing radiation may alter the epigenome (Bernal et al. 2013; Franco et al. 2008), although the modulation of Gpx2 and Gpx4 gene expression can exclude the epigenetic modulation of their gene promoters.

An alternative explanation is the development, during the long culture time at LRE, of (a) mutant(s) with less efficient defence mechanisms (possibly leading to higher radiosensitivity) than the original cells that could have some advantages in cell growth. Since the selection by chance of such mutants is highly unlikely given the consistent results obtained for the replicate cultures, this hypothesis implies that they should occur in all the cultures grown at LRE, i.e. that mutants arise from an environmental radiation-induced selection.

On the whole, the results obtained in this and previous studies carried out under the Gran Sasso mountain (Satta et al. 1995, 2002; Carbone et al. 2009, 2010) suggest that RRE might work as a trigger of defence mechanisms in V79 cells, giving them a higher degree of defence against endogenous damage as compared to cells grown at LRE. The finding that cells grown at LRE for 10 months cannot restore the original enzymatic activity after 6 months of growth at RRE independently of gene expression, suggests that there is a complex interaction of different gene products, including SBP1, with the possible involvement of post-transcriptional and/or metabolic regulation.

The results hereby described underline that the interaction of living matter with very low doses of radiation is an important issue not only for the perspective of radiation protection but also for a better understanding of the evolution of life on earth. In particular, they corroborate the hypothesis that environmental radiation contributes to the development and maintenance of defence mechanisms in organisms living today.

Acknowledgments The authors wish to thank Eng. M. Balata and Dr. L. Ioannucci for their contribution to the logistics of the experiment at INFN-LNGS. Thanks are also due to F. Antonelli, Ph.D., for her help with cell culture, to M. Pinto, Ph.D., for the ^{222}Rn and daughters dose calculation, to Dr. C. Nuccetelli for the measurements

of ^{40}K inside the cells and to Mr. A. Calicchia for the TLD measurements. Thanks are also due to Mr. M. Sabatini for figures preparation and to Ms M. Brocco for the English editing of the manuscript. We gratefully acknowledge Prof. P. Paggi for the discussions and suggestions during the writing of the manuscript.

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