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Effect of cryo-preservation on the response of different biological systems to γ-ray exposure: A feasibility study

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Summary. — Cryopreservation of cells, tissues or organisms (*i.e.* the storage at liquid-nitrogen or nitrogen vapour temperature) offers the most secure form of preservation, allowing cells to be maintained unaltered for a long time and preventing them from ageing. On the other hand, the multi-year exposure of cryo-preserved cells (like: stem and germ cells) to the environmental γ-ray background might induce a radiation-damage accumulation, due to the inhibition of cellular repair mechanisms, and contribute to cancer or non-cancer pathology risk assessment when such cells should be transplanted in individuals. To investigate the effect of the cryopreservation on the cell response to radiation exposures, the response of different biological systems (bacteria and mammalian cells) to γ-rays has been evaluated after

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irradiation in cryo-preserved and culture conditions, as a function of dose and dose-rate, in terms of a variety of cellular and molecular end-points.

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PACS 87.53.Ay – Biophysical mechanisms of interaction.
PACS 87.53.Bn – Dosimetry/exposure assessment.

1. – Introduction

Ionizing radiation can induce DNA strand breaks and mutations that, depending both on damage extent and cell ability to repair it, can finally produce: 1) correct DNA repair produced by DNA repair systems; 2) incorrect DNA repair able to generate DNA point mutation, chromosomal translocation or deletion; 3) programmed cell death, produced by apoptotic cell mechanisms controlling cell integrity [1-5]. In particular, it is known that ionizing radiation can promote the generation of tumour cells and cell ageing.

On the other hand, in cell biology laboratories as well as in clinic practice, a well-established procedure to store for future use biological systems take profit of controlled low-temperatures (cryo-preservation), an expedient to slow down the ageing process of cells or organisms (that have to be maintained unaltered for a long time). Cryopreservation of cells, tissues or organisms can reach in some cases the halt of the metabolic activities (below $-130\,^{\circ}$C or 143 K) and the storage in liquid nitrogen ($-196\,^{\circ}$C or 77 K) or in nitrogen vapour ($-150\,^{\circ}$C or 123 K) offers the most secure form of preservation. However, it is important to consider that cryopreserved biological material can repair the damages produced by ionizing radiations only after defrosting a situation that can interfere with the optimal DNA repair and apoptotic induction. Death process can be rapid and passive (necrosis) due to a strong environmental insult (bad freezing or defrosting, chemical effects of cryoprotectants such as DMSO), or active and slow (apoptotic) and regulated by a homeostatic control mechanism that eliminates cells potentially dangerous to its organism (*i.e.* damaged cells that may become tumour cells).

Information from cryocrystallography, in which biological macromolecules are subjected to radiations for determining their tridimensional structure, suggest that very low temperatures (100 K) considerably reduce (even if not completely block) damages induced by X-rays [6]. Although a lot of experiments have demonstrated that ionizing radiations can induce cell death, tumours and ageing in living cells in a radiation dose-dependent manner, little information is available regarding the response of cryopreserved cells.

The present paper reports the preliminary data gathered in the framework of a “feasibility study” project (named: CRIORAD, CRyogenic preserved biological systems and Ionizing RADiation) designed to investigate the response of cryo-preserved (at liquid-nitrogen or nitrogen vapour temperature) mammalian cells or bacteria to ionizing radiation as a function of dose and dose-rate, in terms of a variety of cellular and molecular end-points.

In particular, CRIORAD plans to compare the response of frozen and non-frozen living cells to γ-rays at different dose levels in order to mimic the multi-year exposure of cryo-preserved cells (like: stem and germ cells) to the environmental γ-ray background and then to contribute to cancer or non-cancer pathology risk assessment when such cells should be transplanted in individuals.

The feasibility study was intended to define and optimize the experimental approaches to face the posed problem and to gather preliminary data on the response of the chosen biological systems to gamma exposures.

In order to study the effect of cryopreservation on the biological response to γ-rays, different biological systems have been used, with different radio-sensitivity: bacteria (*Escherichia coli*), which are highly radiation resistant; Chinese hamster V79 cells, radioresistent cells widely used as reference in cell radiobiology of ionising radiation investigations; human peripheral blood mononuclear cells induced to proliferate, in order to mimic the stem cell behaviour in transplantation.

Biological response to γ-ray exposure has been evaluated in terms of mutation induction and/or cell death (clonogenic death and apoptosis), depending on the specific biological system used. Radiation response of each biological system has been compared after irradiation in cryo-preserved or culture state.

In the present paper the experimental conditions and biological procedures that have been developed and used in this study are reported as well as the preliminary results obtained.

2. – Materials and methods

2·1. *Bacteria cultures*

Cultivation and freezing of bacteria

Enteropathogenic *E. coli* (EPEC strain, characterized by the presence of eaeA and bfpA genes by PCR) was obtained from a human patient affected by enteritis; bacteria were cultured in LB broth at 37 °C. After 20 hours incubation, final concentration was evaluated spectrophotometrically and confirmed by counting CFU (colony forming units). In these experimental conditions bacteria growth (growing samples) ranged from 1 to 2×10^9/ml. Aliquots containing 16×10^6/ml cells were resuspended in freezing solution (10% glycerol in 90% LB Broth), and finally stored in liquid nitrogen until use (frozen samples).

Mutant selection system

Mutant bacteria have been selected by using D-cycloserin [7]. The Minimal Inhibitory Concentration (MIC) of antibiotic was obtained by growing bacteria in liquid media containing serial dilution of D-Cycloserin, ranging from 4 mM to 0.1 mM. Since D-cycloserin MIC was 1 mM, irradiation experiments for selection of mutants were performed by using 1.5 mM D-cycloserin. The number of spontaneous mutations in experimental conditions was 25 to 10^6 live bacteria, both in frozen samples and in growth media.

Mutants in bacteria occur typically at a rate of 10^{-6}/gene/ replication [8].

Killing effect of radiation on frozen and non-frozen E. coli

Frozen bacteria (as previously described) and cells growing in LB Broth have been separately irradiated, with γ-rays from ^{137}Cs source, for a total of 4 hours and 30 min, corresponding to 601 Gy. Samples were irradiated respectively in liquid nitrogen and at room temperature. Cells were harvested at 20 min intervals (corresponding to 45 Gy) and cultured in LB agar to evaluate the viability by counting CFU. Control samples from frozen and non-frozen cultures were plated before irradiation.

The obtained results clearly demonstrated that bacteria are killed after 40 minutes of irradiation (corresponding to 90 Gy).

Mutagenesis analysis on frozen and non-frozen bacteria

Frozen samples. Cryotubes containing 16×10^6/ml *E. coli* have been irradiated with γ-rays from Cs-137 source, for a total of 30 min, corresponding to 67 Gy (samples were irradiated in a dose-fractioned manner: 2 minutes γ-ray exposures, and 1 min interval). After irradiation, 100 μl aliquots have been plated in 40 LB agar plates containing 1.5 mM D-cycloserine, and incubated 16 hours at 37 °C. Aliquots from the same samples have been plated in 30 LB agar plates to evaluate viability of bacteria.

Non-frozen samples. Exponentially growing *E. coli* were diluted in LB broth at 16×10^6/ml concentration. The number of bacteria was confirmed by plating diluted samples in LB agar. A tube containing the same amounts of cells has been irradiated for a total of 30 min, corresponding to 67 Gy (samples were irradiated in a dose-fractioned manner: 2 minutes γ-ray exposures, and 1 min interval). After irradiation, a total of 100 μl aliquots have been plated in 40 LB agar plates containing 1.5 mM D-cycloserine, and incubated 16 hours at 37 °C. Aliquots from the same samples have been plated in 30 LB agar plates to evaluate the viability of bacteria.

2˙2. *Peripheral blood mononuclear cells*

Isolation, freezing and culture of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were obtained from freshly collected blood samples of a healthy male donor (Transfusion Center of San Salvatore Hospital, Pesaro, Italy). PBMC were isolated with Ficoll-Histopaque ($d = 1.077$ g/ml), and then irradiated at room temperature and/or frozen in liquid nitrogen at −196 °C. Briefly, cells have been resuspended in a freezing solution (50% FBS, DMSO 10%, 40% RPMI 1640), stocked into 2 ml vials with 2–5 $\times 10^6$ cells/ml and frozen at −196 °C.

Killing effect of radiation on frozen and non-frozen PBMC

Frozen or non-frozen PBMC were irradiated with 0.3, 0.9 and 3.0 Gy of ^{137}Cs γ-rays, either in single or fractionated doses (delivered in 3 doses separated by 3 hours). After thawing, cells have been analysed to evaluate: 1) cell mortality, soon after thawing by means of trypan blue exclusion and light microscopy; 2) apoptotic process, soon after thawing and after 24, 48 and 72 hours of incubation (37 °C, 5% CO_2) with or without phytohemagglutinin (PHA) stimulation. Cells have been fixed in 70% cold ethanol, stained with propidium iodide and analysed by flow cytometry [5]. The reported percentages were obtained on the basis of at least 40000 cells. Apoptotic cell death induced by ionizing radiation typically involves DNA damaged cells and is revealed by the hypodiploid peak [5].

2˙3. *Chinese hamster V79 cells*

Cells and culture conditions

V79 cells, derived from Chinese hamster embryonic lung fibroblasts, were used. The cells were grown in Eagle's Minimum Essential medium with Dulbecco's modification (D-MEM), supplemented with 10% heat-inactivated foetal bovine serum and incubated in a humidified incubator at 37 °C in atmosphere of 5% CO_2 in air. Cells, coming from the same cultured cell population, were expanded in order to obtain a number of cells sufficient to create a stock for replicating the irradiation experiments several times, and then frozen in cryo-vials (2×10^6 cells/vial).

Irradiation conditions

V79 cells in cultured and frozen conditions were irradiated with different doses (0.5, 1, 3 Gy) of γ-rays. Gamma irradiations were performed at Co-60 gamma beam of CNR-FRAE (at INFN-LNL) with a dose rate of 1 Gy/min and at Cs-137 gamma beam of Dept. of Oncology (University of Padova) with a dose rate of 3 Gy/min. Cultured cells were thawed and kept cycling for 15 days before each irradiation experiment, and were irradiated in monolayer on T25 flask; 3 flasks per dose (with 2.4×10^6 cells/flask) were irradiated. Frozen cells were irradiated in vial; 3 vials were irradiated for each dose. An appropriate set-up was developed for irradiation of frozen cells directly in the cryovials maintained in liquid nitrogen (LN2). This mainly consists of a thermal insulator material block (polystyrene) housing a LN2 container for hosting the cryovials at the adequate temperature (approximately, $-180\,^\circ$C) during irradiation and transfer from the Radiobiology Lab to "gamma beam" facility (and back). Appropriate irradiation geometry has been defined at the "gamma beam" facility to guarantee the same irradiation condition (dose-rate; dose) to frozen or non-frozen cells.

Cell survival and HPRT mutation frequency measurement

After irradiation the non-frozen (cycling) cells were detached and pooled. For each dose, including the 0 Gy (sham irradiated samples), an aliquot of the cell suspension was plated at the appropriate density in 6-cm Petri dishes for survival determination. The remaining cells were subcultured every 48 h to allow the phenotypic expression of mutation at the hypoxanthine-guanine phosphoribosyl transferase (*Hprt*) locus. After 7 days of growth at $37\,^\circ$C, the cells for survival evaluation were fixed and stained, and colonies with > 50 cells were counted as survivors. On days 6th, 8th, 10th after irradiation, 2.5×10^6 cells were seeded for the determination of *Hprt* mutation frequency by measurement of mutants growing in 6-thioguanine ($5\,\mu$g/ml) selective medium. At the same time cells were tested for determining the cloning efficiency. The induced mutation frequency for each experiment was evaluated as $M - M_0$, where M is the number of mutants per viable cell averaged from 6th to 10th day after irradiation and M_0 is the background frequency determined in the same way from the unirradiated cells (experimental control).

After irradiation the frozen cells were thawed and then the same biological protocols as for the cultured cells were used for survival and mutation frequency evaluations.

3. – Results

3˙1. *Bacteria*. – The mutation rates in frozen and non-frozen samples after 67 Gy of γ-ray irradiation have been evaluated by counting CFU in 1.5 mM D-cycloserine agar plates. The number of mutants refers to viable cells in the same sample. Results obtained confirmed that the used radiation doses are not bactericidal, and do not inhibit cell growth during irradiation.

Table I reports the average mutation rate in 10^5 *E. coli* in four independent experiments.

3˙2. *Peripheral blood mononuclear cell*. – Apoptotic cell death of frozen PBMC induced by γ-rays increases with time and dose of radiation. In particular, frozen PBMC have the highest specific cell death (% cell death of irradiated cells $-$% of cell death of non-irradiated cells) after 72 hour of incubation without mitogenic stimulation (fig. 1).

Differently, PHA stimulation of frozen PBMC produces the highest percentage of specific cell death induced by 0.3 Gy after 48 hours of incubation. Surprisingly, PHA

TABLE I. – *First column: the experiments; second column: the growing bacteria; third column: the frozen bacteria. The numbers represent the average mutation rate in* 10^5 *E. coli after exposure to* 67 *Gy of* γ*-rays.*

	Non-frozen bacteria	Frozen bacteria
Exp.1	43 ± 12	15 ± 7
Exp.2	70 ± 26	36 ± 7
Exp.3	48 ± 25	24 ± 13
Exp.4	83 ± 31	24 ± 10

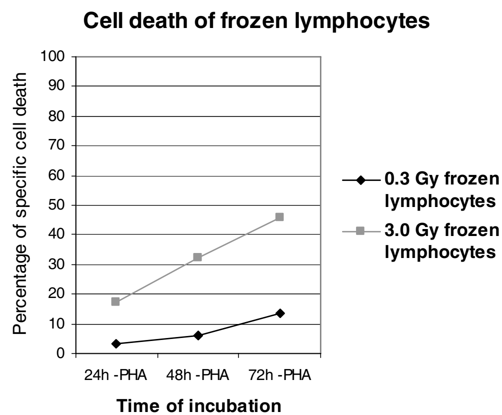

Fig. 1. – Percentage of specific cell death of frozen PBMC after single 0.3 Gy or 3.0 Gy dose radiation (without mitogenic stimulation).

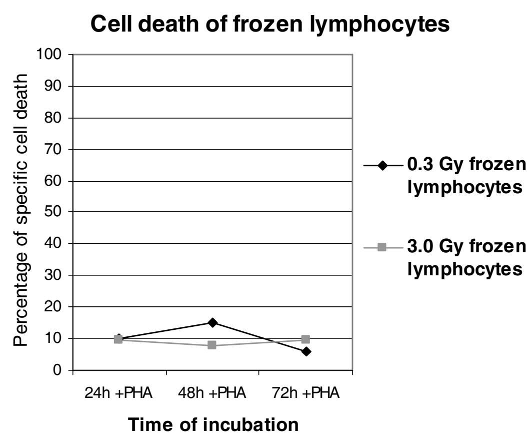

Fig. 2. – Percentage of specific cell death of frozen PBMC after single 0.3 Gy or 3.0 Gy dose radiation with mitogenic stimulation (PHA).

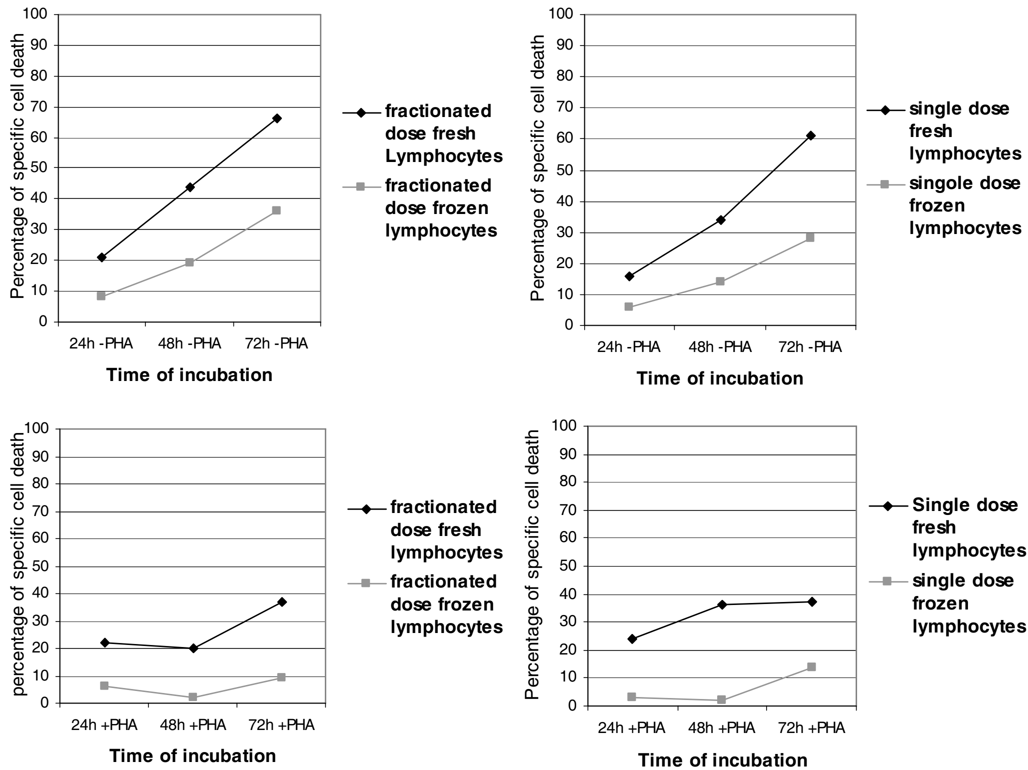

Fig. 3. – Differences of specific cell death on non-frozen (fresh) or frozen PBMC after single 0.9 Gy or 3×0.3 Gy dose radiation with or without mitogenic stimulation (PHA).

stimulation is instead useless at high doses of radiation (3.0 Gy) since it reduces the specific cell death (fig. 2).

Interestingly, irradiation of frozen PBMC produces lower percentage of apoptotic cells compared to fresh PBMC irradiated with the same dose at room temperature and then frozen. This effect is independent of dose fractionation and on mitogenic stimulation (fig. 3).

3·3. *V79 cells.* – Figure 4A shows the dose-response curves for the inactivation of V79 frozen and non-frozen cells irradiated with γ-rays. Both the survival curves have been fitted with the linear model ($S = S_0 \exp[\alpha D]$). The survival curve of non-frozen cells (with slope $\alpha = 0.24 \pm 0.03\,\mathrm{Gy}^{-1}$) is in agreement with data in the literature related to the survival of V79 cells after gamma irradiation [9, 10]. The curve follows a linear behaviour and not a linear-quadratic one ($S = S_0 \exp[\alpha D + \beta D^2]$) as in the literature due to the limited number of data points here considered. Survival of frozen cells decreases more slowly with dose than for non-frozen cells, the survival curve slope being $\alpha = 0.13 \pm 0.06\,\mathrm{Gy}^{-1}$.

Mutation frequency induced in non-frozen cells increases almost linearly with the dose (fig. 4B) and is in agreement with literature data [9,10], in the dose range here considered. After irradiation of frozen cells the mutation frequency increases almost linearly with the dose up to the dose of 1 Gy, with a behaviour similar to the one of the non-frozen cells in the same dose range. The mutation frequency induced by a dose of 3 Gy in frozen cells

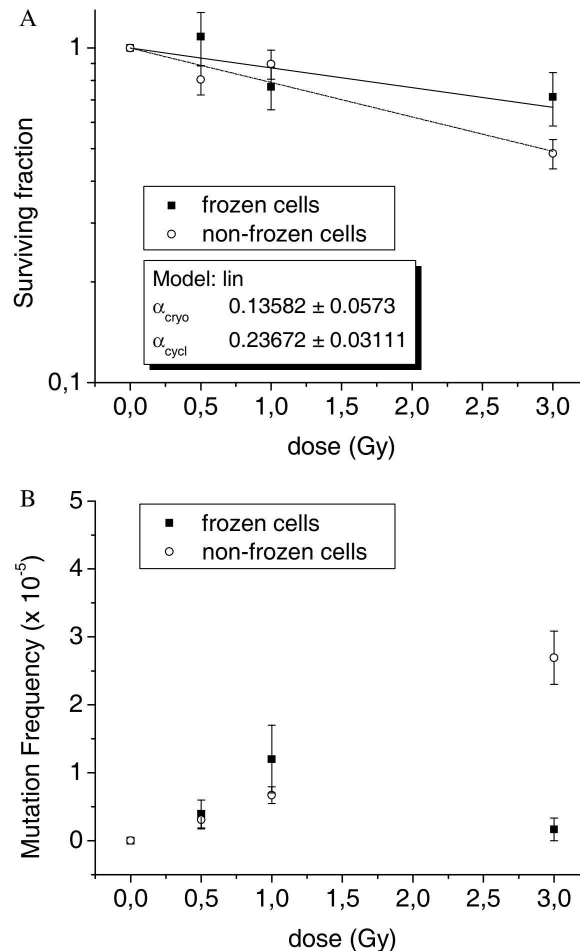

Fig. 4. – Panel A: cell survival curves of frozen and non-frozen V79 cells irradiated with graded doses of γ-rays; panel B: mutant frequency induction at the *Hprt* locus in frozen and non-frozen V79 cells irradiated with graded doses of γ-rays. The dose-response curves (for survival and mutation induction end-points) are the mean of 3 independent experiments. Error bars represent the standard error (or 10% of the mean if the standard error is minor than 10%).

falls down to a level less than the one induced by a dose of 0.5 Gy.

The dose-response curves (for survival and mutation induction end-points) are the mean of 3 independent experiments.

4. – Automatic cell-colony counting system

As a supporting technological activity to the radiobiological investigations, within the project CRIORAD a new in-house automatic system for cell-colony counting in Petri-dishes has been developed. It is based on the elaboration of the digital images of V79 cellular colonies grown on Petri dishes. After acquisition by 8-bit linear scanner, the image is analyzed by an expert system. This is mainly based on the region-growing algorithms for the recognition of the regions of interest (ROI) in the image and a Sanger

neural network for the characterization of such regions. The better final classification is supplied from a Feed-Forward Neural Net. The average counting error (averaged on 150 slabs) can be estimated about 2% [11].

5. – Conclusion

In order to study the effect of cryopreservation on the biological response to γ-rays, three independent parallel investigations have been performed, which differed significantly in several aspects: the biological systems (*E. coli* bacteria; PBMC; V79 cells); the end-points considered to evaluate the radiation response (mutation for bacteria, apoptosis for PBMC, clonogenic death and *Hprt* mutation for V79 cells); the γ-ray irradiation facilities (γ-sources, geometry, dose and dose-rates).

Notwithstanding these differences, the three independent investigations have given coherent results. As regards bacteria, the number of mutants induced by γ-rays is systematically lower in frozen bacteria than in non-frozen ones, showing a protective effect of cryopreservation against radiation damage.

As expected, apoptotic cell death of frozen PBMC induced by gamma radiation increases with time and dose of radiation. Interestingly, frozen PBMC have a reduced cell death compared to non-frozen PBMC, indicating that frozen state protects from cell death induced by ionizing radiation.

This effect might depend on block of free radicals produced by low temperatures [6,12] and/or impairment of apoptosis inducing mechanisms. Although cell freezing partially protects PBMC from cell death, we were able to detect a significant level of apoptosis at the lower dose tested (0.3 Gy, figs. 1, 2) and this may depend on low dose hypersensitivity of PBMC [13].

A similar result has been obtained for V79 cells, where frozen cells seem to be lightly protected with respect to non-frozen cells against clonogenic death and *Hprt* mutations induced by γ-rays. In particular, clonogenic death increases linearly with the dose but more rapidly for non-frozen cells than for frozen cells. As concerns *Hprt* mutation induction, data show that mutation frequency increases with dose over the 0–3 Gy dose range in non-frozen cells, while a similar behaviour is obtained for frozen cells only up to 1 Gy and a fall-down in mutation frequency is found after 3 Gy exposure. This shows that cryopreservation seems to be protective for cells against mutations induced by doses over a 1–2 Gy level, but any effect (protection or enhancement) seems to be induced at lower doses (0.5, 1 Gy).

All the data gathered in this study seem to indicate a protective effect of cryopreservation against the induction of radiation damage. Taking into account the differences among the three investigations, as highlighted here above, this protective effect cannot be ascribed to the experimental method (biological systems, biological end-points, irradiation facilities) but seems to be related to the cryopreservation state.

This result is in agreement with findings by food irradiation practice, which show that a higher dose of γ-rays is necessary for sterilization of frozen food [14]. This effect might depend on the slowing down at very low temperatures of the diffusion of free radicals produced by incident radiations which would imply that radical contribution to radiation damage is reduced or suppressed [6, 12].

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

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