

Fullerenol C₆₀(OH)₂₄ Effects on Antioxidative Enzymes Activity in Irradiated Human Erythroleukemia Cell Line

Višnja BOGDANOVIĆ^{1†}, Karmen STANKOV^{2,3*†}, Ivana IČEVIĆ^{4†}, Dragan ŽIKIĆ⁵, Aleksandra NIKOLIĆ⁶, Slavica ŠOLAJIĆ¹, Aleksandar DJORDJEVIĆ^{4**} and Gordana BOGDANOVIĆ¹

Fullerenol/Radioprotection/Oxidative stress/K562 cell line.

Radiotherapy-induced toxicity is a major dose-limiting factor in anti-cancer treatment. Ionizing radiation leads to the formation of reactive oxygen and nitrogen species (ROS/RNS) that are associated with radiation-induced cell death. Investigations of biological effects of fullerenol have provided evidence for its ROS/RNS scavenger properties *in vitro* and radioprotective efficiency *in vivo*. Therefore we were interested to evaluate its radioprotective properties *in vitro* in the human erythroleukemia cell line. Pre-treatment of irradiated cells by fullerenol exerted statistically significant effects on cell numbers and the response of antioxidative enzymes to X-ray irradiation-induced oxidative stress in cells. Our study provides evidence that the pre-treatment with fullerenol enhanced the enzymatic activity of superoxide dismutase and glutathione peroxidase in irradiated K562 cells.

INTRODUCTION

Polyhydroxylated fullerenes have been recently recognized as exogenous redox balance modulators, capable to exert anti-oxidative effects in both *in vitro* and *in vivo* systems.¹⁾ Thus, the antioxidative activity of fullerenols is their most exploited property in medicinal chemistry.

Reactive oxygen/nitrogen species (ROS/RNS), generated by ionizing radiation, have been implicated in its harmful effects on living tissues, inducing apoptosis of malignant

and normal cells, by triggering mitochondrial permeability transition pore opening.²⁾ Application of radiotherapy for the control of cancer, either alone or in conjunction with chemotherapy, is often limited by normal tissue toxicity including haematopoietic toxicity.

Our recently published results showed the *in vivo* radioprotective efficiency of fullerenol in irradiated rats, as well as its nitric oxide (NO) – quenching activity in both *in vivo* and *in vitro* systems.^{3–5)} Therefore, we were particularly interested in fullerenol *in vitro* effects on malignant cultured cells of human erythroleukemia. The aim of our study was to estimate the number, cell viability and colony-forming capacity of irradiated cells, treated by fullerenol, together with the response of anti-oxidative enzymes to X-ray radiation induced oxidative stress in these cells.

Exposure of cells to ionizing radiation leads to the formation of ROS that are associated with radiation-induced cytotoxicity. The antioxidative enzyme superoxide dismutase (SOD) catalyzes the dismutation of the superoxide anions into hydrogen peroxide.⁶⁾ SOD is known as a factor conferring increased protection from lethal irradiation to haematopoietic cells.⁶⁾

The activity of γ -glutamyltransferase (GGT) is frequently upregulated in tumor cells after oxidative stress and may thus increase the availability of amino acids needed for biosynthesis of the antioxidant glutathione (GSH). As irradiation of tumor cells enhances oxidative stress, we investigated whether such treatments can modulate the enzyme level of GGT in K562 cells.⁷⁾

*Corresponding author: Phone: +33-1-60-87-83-77,

Fax: +33-1-60-87-84-85,

E-mail: stankov@cng.fr

**Corresponding author: Phone: +381-21-48-52-759,

Fax: +381-21-455-662,

E-mail: dvadj@ih.ns.ac.yu

¹⁾Institute of Oncology, Department of Experimental Oncology, Sremska Kamenica, Serbia; ²⁾Medical faculty, Department of Biochemistry, Novi Sad, Serbia; ³⁾Present address: CEA/Institut de Génétique, Centre National de Génotypage, 2 rue Gaston Crémieux, bâtiment G2, CP 5721, 91057 Evry Cedex, France; ⁴⁾Faculty of Science, Department of Chemistry, Trg Dositeja Obradovica 3, 21000 Novi Sad, Serbia; ⁵⁾Faculty of Agriculture, Department of Animal Science, Novi Sad, Serbia; ⁶⁾Institute for Biological Research “Sinisa Stankovic”, Belgrade, Serbia.

[†]These authors contributed equally to this work.

Abbreviations: ROS, reactive oxygen species; RNS, reactive nitrogen species; NO, nitric oxide; O₂⁻, superoxide; GGT, gamma – glutamyl transferase; SOD, superoxide dismutase; GPX, glutathione peroxidase; GSH, glutathione; NOS, nitric oxide synthase

doi:10.1269/jrr.07092

In order to estimate the possible radioprotective effects of fullereneol on irradiated K562 erythroleukemia cell line, we measured the activity of GGT and SOD in K562 cells, together with the activity of glutathione peroxidase (GPX), a key enzyme for the cellular defense against ROS.

MATERIALS AND METHODS

Fullereneol $C_{60}(OH)_{24}$ (Fig. 1) was synthesized and characterized by Djordjević *et al.*, from polybromine derivative $C_{60}Br_{24}$, which was synthesized in reaction of C_{60} in Br_2 with $FeBr_3$ as catalyst.^{8,9)} Obtained results are very similar to those published by Chen *et al.*¹⁰⁾ Fullereneol $C_{60}(OH)_{24}$ was dissolved in distilled water and was adjusted to pH = 7.4 with phosphate buffer immediately before use.

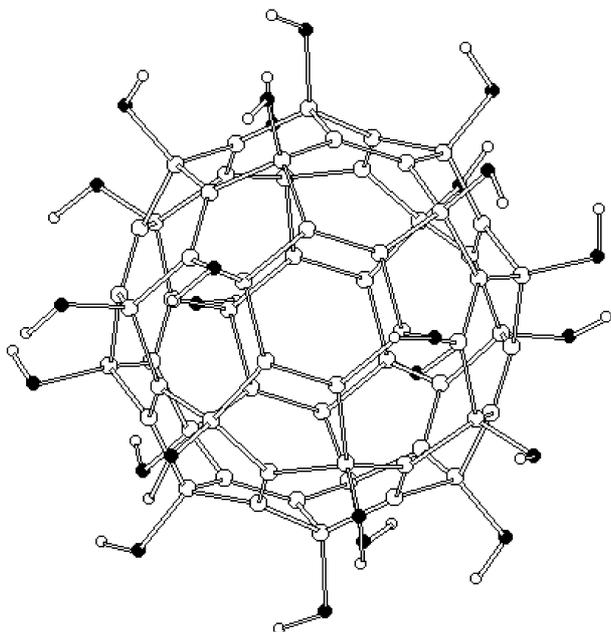


Fig. 1. Schematic diagram of water soluble fullerene derivative fullereneol $C_{60}(OH)_{24}$.

Human erythroleukemia K562 cell line was cultured in 25 cm^2 flasks (Costar, USA) in 10 ml of RPMI 1640 medium (Sigma, USA), supplemented with 2 mM L-glutamine, 10% FCS (v/v) (Veterinary institute Novi Sad - NIVNS), 100 IU/ml penicillin (ICN, USA) and 100 μ g/ml streptomycin (ICN, USA) at 37°C in fully humidified atmosphere with 5% CO_2 .

For the experiments with irradiation and fullereneol pre-treatment, K562 cells were seeded in Petri dishes, 5×10^5 cells in 5 ml of RPMI 1640 with 10% FCS. Water dissolved fullereneol (pH = 7.4) was added to cells in medium, in final concentration of 10 μ M, 30 minutes prior to X-ray irradiation. Irradiation was carried out 24h post-planting time at 25°C. K562 cells were exposed to 24 Gy single dose of X-rays, using radiotherapeutic instrument LINAC Mevatron

MD 7475 (Siemens, Germany) (10 MV X-rays), at a dose rate of 3 Gy/min.

The experimental conditions applied in this study, concerning the selection of fullereneol and irradiation dose, are based on our preliminary experiments of dose-dependent effects on survival of fullereneol pre-treated cells subjected to irradiation. This *in vitro* screening study, performed on a panel of human malignant hematopoietic cell lines (K562, Raji, HL60 and PC-MDS cells), showed that the 10 μ M of fullereneol and 24 Gy single dose of X-rays, induced the optimal K562-cell specific response.¹⁾

The experimental design consisted of the following sets: a/ control non-irradiated cells; b/ non-irradiated cells pre-incubated with 10 μ M of fullereneol; c/ X-ray (24 Gy) irradiated cells; d/ X-ray (24 Gy) irradiated cells, pre-incubated with 10 μ M of fullereneol.

K562 cells were passaged 24h before the experiment and were maintained in medium for next 1, 24 and 48 hours. Therefore, each of the parameters in four experimental sets was estimated in time-dependent manner after the irradiation.

In each of the experimental sets, the number of cells was determined using the dye exclusion test (DET).¹¹⁾ Trypan blue exclusion test was performed by mixing 200 μ l of cell suspension (1×10^5 /ml) with an equal volume of 0.3% Trypan blue solution (Sigma, St Louis, MO) in PBS. After 5 minutes of incubation at room temperature, the number of cells excluding Trypan blue (unstained) was determined, using a Burker Turk hemocytometer to estimate the cell survival in all experimental sets.

Morphological alterations in surviving fraction of control and irradiated K562 cells were followed and documented by use of light microscope (100 \times magnification level) (Olympus BX51), using May-Grünwald Giemsa (MGG) cell-staining method. Cells were prepared by centrifugation (Shandon cytopsin 3, Thermo Fisher Scientific, USA) (800 rpm, 10×10^3 cells/0.1 ml in each sample chamber). The cellular area of 100 cells in each experimental set was measured with optical resolution of 6.57 pixels, using Scion image software.

Following irradiation, the cells were assayed for viability by DET, and then transferred to viscous medium for the colony forming unit quantitative test. The irradiated cells (3.5×10^3 K562 cells per well) were plated in medium consisting of RPMI 1640, 10% FCS in 0.8% methylcellulose (Dow Chemical Co.). Colonies were scored following incubation at 37°C with 5% CO_2 for 10 days. Only those aggregates containing more than 50 cells were scored as colonies. The K562 cell line formed colonies only in methylcellulose and grew in suspension culture in the absence of detectable adherence.¹²⁾

All enzyme activity analyses were performed in cytosolic cell fraction, in supernatant obtained by ultrasonication (Soniprep 150 MSE) (10 minutes at 10 000 rpm at 4°C) and

kept at -80°C . The enzyme activities of γ -glutamyl transferase, total superoxide dismutase and glutathione peroxidase were determined by kinetic method.¹³⁻¹⁵ The enzyme activity is expressed as units per 10^6 of cells.

All the experiments were done in triplicate and enzyme analyses in quadruplicate. Results are expressed as mean \pm standard deviation of the mean value (SD). Differences among means were tested for statistical significance by student's two-tailed t-test and one-way ANOVA test. Statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

The survival rates of K562 cells, determined by DET analysis, showed that the X-ray irradiation (24 Gy), induces cell death of 50% of cells, at 24h post-irradiation time (Fig. 2). Our experiments showed that K562 cell line is sensitive to irradiation of 24 Gy. This result is comparable to the results showing the double strand damage and massive apoptosis at 24h post-irradiation time.^{16,17} At 48 h after irradiation, the number of K562 cells was increased, indicating that the survived fraction of cells continued to proliferate. This effect was more pronounced in fullerenol pre-treated cells. The fullerenol applied alone does not induce the cytotoxic effects, which is in accordance with other *in vitro* studies.¹⁸⁻²⁰

Cell morphology was estimated by means of computer-assisted image analysis. In the cellular set exposed to 24 Gy, we found morphological changes consisting of significantly larger cell size and the appearance of numerous vacuoles throughout the cytoplasm, which was absent in control cellular set, as well as in the fullerenol pre-treated irradiated K562 cells at 24 h time point (data not shown).

The initiation and execution of cell apoptosis program is a key determinant of irradiation response in tumor cells. In cell culture studies it is defined as loss of the ability to form colonies, due to growth arrest and cell senescence.²¹ Our experiments showed statistically significant decrease in colony forming ability of irradiated cells (data not shown). It is interesting to note that the pre-treatment with fullerenol partially prevented this effect in irradiated cells.

The activity of GGT is frequently upregulated as a consequence of an irradiation-induced acute oxidative stress.⁷ Our results showed that the activity of GGT in irradiated K562 cells was significantly increased, in time-related manner, being 4 fold increased at 24 h post-irradiation time, and 2.8 fold at 48 h post-irradiation time (Fig. 3). The fullerenol pre-treatment, significantly reduced the levels of GGT in irradiated cells, indicating the important role of fullerenol for maintenance of redox homeostasis.¹⁸ The activity of GGT is increased after the exposure to NO, thus, the ability of fullerenol to scavenge NO is in accordance with decreased GGT activity in fullerenol pre-treated irradiated cells.^{5,7}

The increase in SOD activity has been postulated as one of the possible mechanisms of radioprotection for hematopoietic cells.²² Our data are showing the significant increase of total SOD levels in irradiated cells, at 1 hour post-irradiation time (Fig. 4). More interestingly, we observed 2 and 3 fold elevated SOD levels at 24 and 48 h time points, respectively, in fullerenol pre-treated non-irradiated cells, and 2 fold elevated SOD activity in fullerenol pre-treated irradiated cells at 24 hours post-irradiation time. These results are in accordance with fullerenol ROS scavenging activity, observed in both *in vivo* and *in vitro* systems.²³

The administration of fullerenol before irradiation induced a significant increase in the activities of both SOD

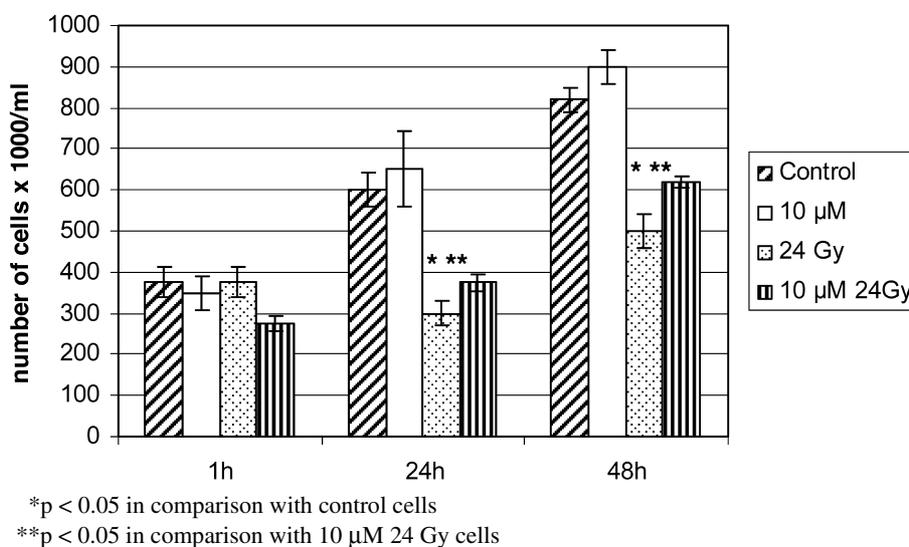


Fig. 2. Number of K562 cells in different experimental sets: Control: non-irradiated cells; 10 μM : non-irradiated, fullerenol pre-treated cells; 24 Gy: irradiated cells; 10 μM 24 Gy: irradiated, fullerenol pre-treated cells.

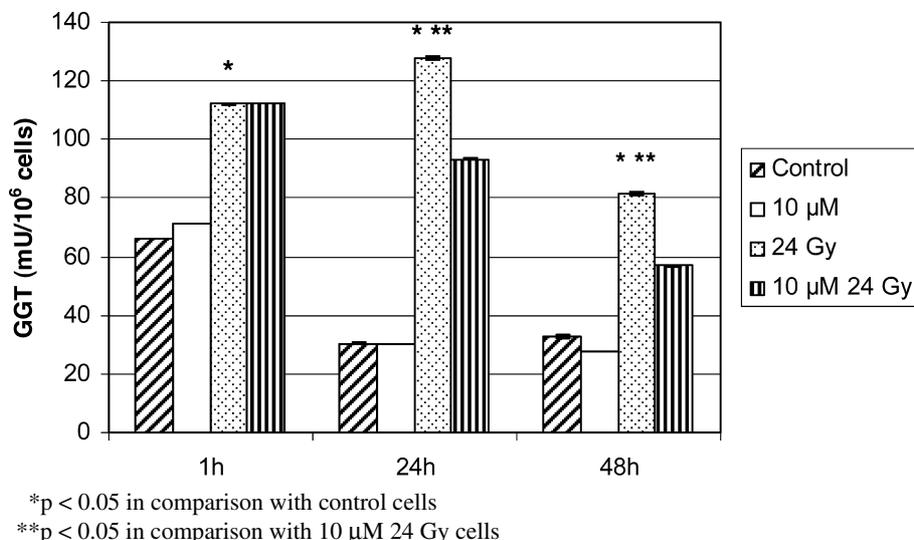


Fig. 3. Gamma-glutamyltransferase (GGT) enzyme activity (mU/10⁶ cells) in K562 cell line in different experimental sets: Control: non-irradiated cells; 10 μM: non-irradiated, fullereneol pre-treated cells; 24 Gy: irradiated cells; 10 μM 24 Gy: irradiated, fullereneol pre-treated cells.

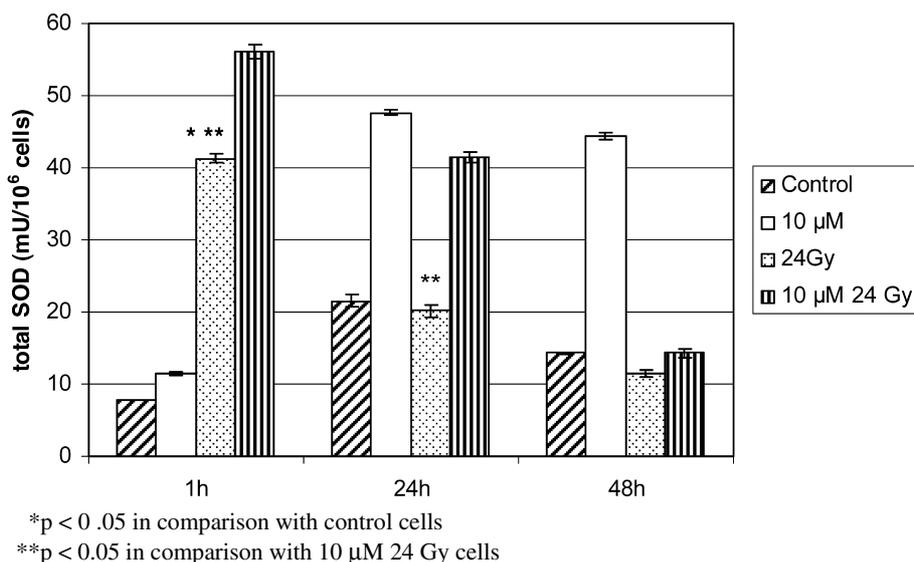


Fig. 4. Total superoxide-dismutase (SOD) enzyme activity (mU/10⁶ cells) in K562 cell line in different experimental sets: Control: non-irradiated cells; 10 μM: non-irradiated, fullereneol pre-treated cells; 24 Gy: irradiated cells; 10 μM 24 Gy: irradiated, fullereneol pre-treated cells.

and GPX. This effect was followed by preserved cell number and colony forming capacity in the same experimental set. Irradiation effects on cellular redox balance are mediated by dramatic depletion of GSH content and consecutive decline of SOD and GPX activity, due to ROS generation. Our results are comparable with those of the already published study, showing an increase of SOD and GPX activity after pre-treatment of irradiated skin of experimental animals with ascorbic acid, which has been reported to be a good ROS scavenger and radioprotective agent.²⁴⁾ We may

hypothesize that the fullereneol pre-treatment prevented the deleterious effects of ROS, directly by increasing the antioxidant enzyme activities of irradiated cells.

Our hypothesis, concerning the possible mechanisms responsible for the increased activity of SOD in K562 cells pre-treated by fullereneol, may be viewed in light of the possibility that the NO scavenging - fullereneol activity may prevent superoxide consumption in the reaction of formation of peroxynitrite anion, concomitantly increasing the O₂^{•-} concentration, and increasing SOD activity as a consequence of

superoxide excess.

Our results *in vivo* are showing efficient scavenging of NO by fullerenol, further supported by the results of other authors showing fullerene derivatives-induced inhibition of all three forms of NOS.^{5, 25)}

Nitric oxide (NO) reacts with superoxide ($O_2^{\cdot-}$) at diffusion controlled rates, competing with endogenous SOD for substrate, which is determined by bimolecular rate constants ($1.9 \times 10^{10} M^{-1} s^{-1}$ and $2 \times 10^9 M^{-1} s^{-1}$ respectively).²⁶⁾ It has been shown that micromolar amounts of SOD compete effectively with NO for superoxide and that the inhibition of nitric oxide synthase (NOS) activity or treating cells with NO scavengers may induce SOD activity.²⁷⁾

Concentration of fullerenol should exceed that of superoxide, in order to achieve the effective competition in the reaction of the formation of peroxynitrite anion. Superoxide cellular concentrations under normal, nonstressed physiological conditions are less than nanomolar.^{27,28)} Therefore, it should be necessary to measure the intracellular concentration of fullerenol, always taking into consideration the cell/tissue specificities as well as the differences in water solubility or tissue permeability of different fullerene derivatives.

It would be very interesting to carry out further kinetic studies in order to prove that by NO scavenging, fullerenol may prevent the formation of peroxynitrite anion, a reactive and short-lived species that promotes oxidative molecular and tissue damage, due to fullerenol effects on decreasing the bioavailability of NO, one of the peroxynitrite precursor radical species.²⁹⁾

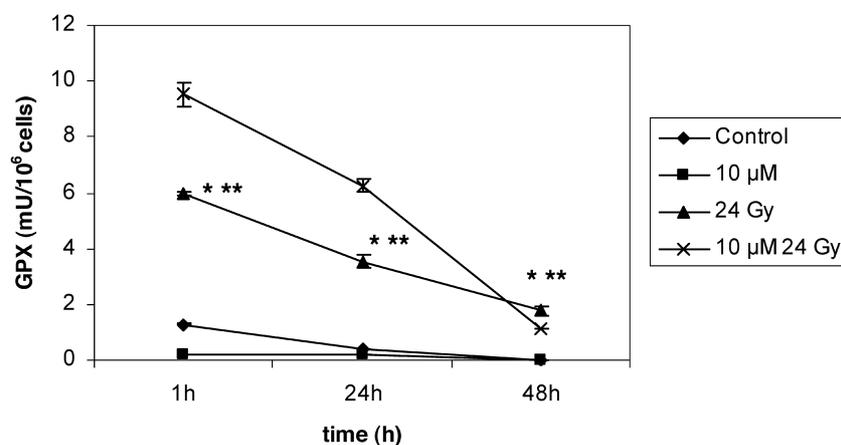
Further investigation remains to be performed in order to support the hypothesis of effective competition of fullerenol and superoxide for nitric oxide, consequently enhancing the SOD activity due to excess of superoxide. This hypothesis

of the fullerenol effects, attributed to preventing the interaction of nitric oxide with superoxide, must be interpreted with caution and require further investigation.

Glutathione (GSH) acts as a co-factor with the enzyme glutathione peroxidase (GPX) to detoxify ROS in irradiated cells and tissues.^{24,30)} Therefore, it would be of fundamental importance to characterize the substances, which may be safely administered as radio-protectors, in order to achieve maximal tumor suppression during radio therapy, with minimal collateral damage to normal tissues. GPX is the enzyme required for reduction of hydrogen peroxide, and its activity is reflecting the cellular responses to increased level of ROS.

Fullerenol pre-treatment did not change the control levels of GPX activity (1h time point) (Fig. 5). In contrast, the levels of GPX at 1h time point were higher in both irradiated cellular sets. Following fullerenol pre-treatment and 24 Gy irradiation, the GPX level was significantly elevated at 1h post-irradiation time, in comparison with other experimental sets. This effect of fullerenol pre-treatment in irradiated cells may be partially explained by consecutive activation of GPX due to SOD increased activity, as well as by substrate accumulation in cell cytosole, which inhibits the GPX enzyme active center.³¹⁾

The chemistry of oxidative stress has focused upon generation of hydroxyl radical and superoxide.³⁷⁾ There are many contradictory and intriguing data, concerning protective role of C_{60} derivatives towards ROS/RNS and irradiation-induced oxidative stress.^{7,18,25)} The growing evidence suggests that the fullerenol-induced activity of the enzymes involved in cell resistance to oxidative stress, may be considered as an important mechanism for the maintenance of redox balance and cell survival after irradiation, which is not restricted only to normal cells, according to our results.



*p < 0.05 in comparison with control cells

**p < 0.05 in comparison with 10 μM 24 Gy cells

Fig. 5. Glutathione peroxidase (GPX) enzyme activity (mU/10⁶ cells) in K562 cell line in different experimental sets: Control: non-irradiated cells; 10 μM: non-irradiated, fullerenol pre-treated cells; 24 Gy: irradiated cells; 10 μM 24 Gy: irradiated, fullerenol pre-treated cells.

Our previously published results are showing that fullereneol induces moderate changes in cell cycle distribution, DNA synthesis and mitotic activity of K562 cells, indicating cytostatic rather than cytotoxic activity of fullereneol.¹⁾ Data related to genotoxic properties of water soluble fullerenes are still insufficient. Our *in vitro* study on two human breast cancer cell lines did not show the significant augmentation of sister chromatid exchanges, nor the incidence of micronuclei in fullereneol treated cells compared to control.¹⁾ Therefore, our present data may be discussed in light of our previously published results, showing no genotoxic activity of fullereneol.

The fullereneol capacity to form clusters in water, with a mean size of 100 nm or more, at physiological pH and temperature, may be responsible, in part, for the biological effects we observed.³³⁾ The aim of our ongoing studies is to further support this hypothesis.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Science, Technology and Development, Republic of Serbia, Grant No. 142076.

REFERENCES

- Djordjević, A., Bogdanović, G. and Dobric, S. (2006) Fullerenes in biomedicine. *J BUON*. **11**: 391–404.
- Fruehauf, J. P. and Meyskens, F. L. (2007) ROS: a breath of life or death. *Clin. Cancer Res.* **13**: 789–794.
- Trajkovic, S., Dobric, S., Jacevic, V., Dragojevic-Simic, V., Milovanovic, Z. and Djordjević, A. (2007) Tissue protective effects of fullereneol C₆₀(OH)₂₄ and amifostine in irradiated rats. *Colloids Surf. B: Biointerfaces*. **58**: 39–43.
- Trajkovic, S., Dobric, S., Djordjević, A., Dragojevic-Simic, V. and Milovanovic, Z. (2005) Radioprotective efficiency of fullereneol in irradiated rats. *Materials Science Forum* **494**: 549–554.
- Mirkov, S. M., Djordjević, A. N., Andric, N. L., Andric, S. A., Kostic, T. S., Bogdanović, G. M., Vojinović-Miloradov, M. B. and Kovacevic, R. Z. (2004) Nitric oxide-scavenging activity of polyhydroxylated fullereneol, C₆₀(OH)₂₄. *Nitric Oxide* **11**: 201–207.
- Southgate, T. D., Sheard, V., Milsom, M. D., Ward, T. H., Mairs, R. J., Boyd, M. and Fairbairn, L. J. (2007) Radioprotective gene therapy through retroviral expression of manganese superoxide dismutase. *J. Gene Medicine*. **9**: 244–252.
- Pankiv, S., Moller, S., Bjorkoy, G., Moens, U. and Huseby, N. E. (2006) Radiation-induced upregulation of gamma-glutamyltransferase in colon carcinoma cells is mediated through the Ras signal transduction pathway. *Biochim. Biophys. Acta*. **1760**: 151–157.
- Djordjević, A. (2000) Synthesis of water soluble derivatives of fullerene C₆₀. PhD Thesis, University of Novi Sad, Novi Sad.
- Djordjević, A., Vojinović-Miloradov, M., Petranović, N., Devečerski, A., Lazar, D. and Ribar, B. (1998) Catalytic preparation and characterization of C₆₀Br₂₄. *Fullerenes Sciences & Technology* **6**: 689–694.
- Chen, Y., Cai, R. F., Chen, S. and Huang, Z. E. (2001) Synthesis and characterization of fullerol derived from C₆₀ precursors. *J. Phys. Chem. Sol.* **62**: 99–101.
- Yoshida, T., Kondo, T., Ogawa, R., Feril, L. B., Zhao, Q. L., Watanabe, A. and Tsukada, K. (2007) Combination of doxorubicin and low-intensity ultrasound causes a synergistic enhancement in cell killing and an additive enhancement in apoptosis induction in human lymphoma U937 cells. *Cancer Chemother. Pharmacol.* (2007); doi 10.1007/s00280-007-0503-y.
- Del Cañizo, M. C., Mota, A., Orfao, A., Galende, J., Caballero, M. D., Garcia Marcos, M. A. and San Miguel, J. F. (1996) Value of colony forming unit-granulocyte macrophage assay in predicting relapse in acute myeloid leukaemia. *J. Clin. Pathol.* **49**: 450–452.
- Shaw, L. M. (1983) γ -Glutamyl transferase. In: *Methods of enzymatic analysis*, volume III, Ed. Bergmeyer, H.U., pp. 349–364, Weinheim, Basel.
- Misra, H. P. and Fridovich, I. (1972) The generation of superoxide radical during the autoxidation of hemoglobin. *J. Biol. Chem.* **247**: 6960–6962.
- Beutler, E. (1984) Glutathione Peroxidase (GSH-Px). In: *Red Cell Metabolism: A Manual of Biochemical Methods*, Ed. Beutler E., pp. 74–76, Grune & Stratton, New York.
- Mustonen, R., Bouvier, G., Wolber, G., Stohr, M., Peschke, P. and Bartsch, H. (1999) A comparison of gamma and neutron irradiation on Raji cells: effects on DNA damage, repair, cell cycle distribution and lethality. *Mutation Res.* **429**: 169–179.
- Tabocchini, M. A., Rothkamm, K., Signoretto, C., Risse, J., Sapor, O. and Loblrich, M. (2000) Formation and repair of DNA double-strand breaks in γ -irradiated K562 cells undergoing erythroid differentiation. *Mutation Res.* **461**: 71–82.
- Niva, Y. and Iwai, N. (2006) Genotoxicity in Cell Lines Induced by Chronic Exposure to Water-Soluble Fullerenes Using Micronucleus Test. *Environmental Health and Preventive Medicine*. **11**: 292–297.
- Bogdanović, G., Kojic, V., Djordjević, A., Canadanovic-Brunet, J., Vojinović-Miloradov, M. and Baltic, V. (2004) Modulating activity of fullerol C₆₀(OH)₂₂ on doxorubicin-induced cytotoxicity. *Toxicol. in vitro* **18**: 629–637.
- Kojić, V., Jakimov, D., Bogdanović, G. and Djordjević, A. (2005) Effects of fullereneol C₆₀(OH)₂₂ on cytotoxicity induced by antitumor drugs on human breast carcinoma cell lines. *Materials Science Forum*, **494**: 543–548.
- Roninson, I. B., Brode, E. V. and Chang, B. D. (2001) If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells. *Drug Resist. Updat.* **4**: 303–313.
- Suresh, A., Tung, F., Moreb, J. and Zucali, J. R. (1994) Role of manganese superoxide dismutase in radioprotection using gene transfer studies. *Cancer Gene Ther.* **1**: 85–90.
- Daroczi, B., Kari, G., McAleer, M. F., Wolf, J. C., Rodeck, U. and Dicker, A. P. (2006) *In vivo* radioprotection by the fullerene nanoparticle DF-1 as assessed in a zebrafish model. *Clin. Cancer Res.* **12**: 7086–7091.

24. Chandra Jagetia, G., Rajanikant, G. K., Rao, S. K. and Shrinath Baliga, M. (2003) Alteration in the glutathione, glutathione peroxidase, superoxide dismutase and lipid peroxidation by ascorbic acid in the skin of mice exposed to fractionated γ radiation. *Clin. Chim. Acta.* **332**: 111–121.
25. Bosi, S., Da Ros, T., Spalluto, G. and Prato, M. (2003) Fullerene derivatives: an attractive tool for biological applications. *Eur. J. Med. Chem.* **38**: 913–923.
26. Kinnula, V. and Crapo, J. (2004) Superoxide dismutases in malignant cells and human tumors. *Free Rad. Biol. Med.* **36**: 718–744.
27. Wink, D. and Mitchell, J. (1998) Chemical biology of nitric oxide: Insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. *Free Rad. Biol. Med.* **25**: 434–456.
28. Tyler, D. (1975) Polarographic assay and intracellular distribution of superoxide dismutase in rat liver. *Biochem. J.* **147**: 493–504.
29. Radi, R., Peluffo, G., Noel Alvarez, M., Naviliat, M. and Cayota, A. (2001) Unraveling peroxynitrite formation in biological systems. *Free Rad. Biol. Med.* **30**: 463–488.
30. Stankov, K. and Kovacevic, Z. (1998) Oxidative stress and glutathione-associated antioxidative systems in malignant cells. *Arch. Oncol.* **6**: 109–113.
31. Little, C., Olinescu, R., Reid, G. and O'Brien, P. J. (1970) Properties and regulation of glutathione peroxidase. *J. Biol. Chem.* **245**: 3632–3636.
32. Epperly, M. W., Cao, S., Zhang, X., Franicola, D., Shen, H., Greenberger, E. E., Epperly, L. D. and Greenberg, J. S. (2007) Increased longevity of hematopoiesis in continuous bone marrow cultures derived from NOS1 (nNOS, mtNOS) homozygous recombinant negative mice correlates with radioresistance of hematopoietic and marrow stromal cells. *Exp. Hematol.* **35**: 137–145.
33. Brant, J. A., Labile, J., Robichaud, C. O. and Wiesner, M. (2007) Fullerol cluster formation in aqueous solutions: Implications for environmental release. *J. Colloid Interface Sci.* **314**: 281–288.

Received on September 14, 2007

1st Revision received on October 17, 2007

2nd Revision Received on December 8, 2007

Accepted on December 21, 2007

J-STAGE Advance Publication Date: February 16, 2008