

Size distribution of fullerene nanoparticles in cell culture medium and their influence on antioxidative enzymes in Chinese hamster ovary cells

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Abstract

Fullerenol (C₆₀(OH)₂₄) nanoparticles (FNP) have a significant role in biomedical research due to their numerous biological activities, some of which have cytoprotective and antioxidative properties. The aim of this study was to measure distribution of fullerene nanoparticles and zeta potential in cell medium RPMI 1640 with 10% fetal bovine serum (FBS) and to investigate the influence of FNP on Chinese hamster ovary cells (CHO-K1) survival, as well as to determine the activity of three antioxidative enzymes: superoxide-dismutase, glutathione-reductase and glutathione-S-transferase in mitomycin C-treated cell line. Our investigation implies that FNP, as a strong antioxidant, influences the cellular redox state and enzyme activities and thus may reduce cell proliferation, which confirms that FNP could be exploited for its use as a cytoprotective agent.

Keywords: fullerene, mitomycin C, antioxidative enzyme, CHO K1 cell line.

Available online at the Journal website: <http://www.ache.org.rs/HI/>

With the rapid development of nanotechnology, many kinds of nanomaterials have been and are being used in fields of industry and scientific researches. A wide range of engineered nanoparticles, ranging from 1–100 nm, have been proposed to be used in nanomedicine due to their unique physical and chemical characteristics. Fullerenols are polyhydroxylated fullerenes C₆₀(OH)_x (between 2 > x < 44). Fullerene C₆₀(OH)₂₄ has a diameter of approximately 1 nm with symmetrically arranged hydroxyl groups on the C₆₀ sphere [1]. Fullerene dissolved in water forms polyanion nanoparticles of size mostly between 3 and 100 nm [2,3]. Polyhydroxylated fullerenes, including C₆₀(OH)₂₄, have demonstrated high antioxidative activity in many chemical, *in vivo* and *in vitro* studies [4–7]. Mrdjanovic *et al.* confirmed the antigenotoxic effect of FNP on mitomycin-damaged CHO-K1 cells [8]. *In vitro* and *in vivo* studies have proved fullerene's tissue-protective effect in irradiated human erythroleukemia cell line K562 and organs of rats, due to its antioxidative and radical scavenging activity [9,10]. Results of studies on healthy and tumor-bearing rats, treated with a single high dose

of doxorubicin (DOX), imply the potential tissue-protective role of FNP [11–18].

Internalization of nanoparticles into live cells is closely related to their potential application, function and cytotoxicity. It is known that cellular uptake and the processes of cellular delivery are influenced by various factors, such as: physicochemical properties of nanoparticles (chemical composition, size, shape and surface charge), concentration of nanoparticles, incubation time, the type of cells, etc. [19].

Mitomycin C (MMC) is an antitumor quinone that undergoes reductive metabolism to generate reactive electrophilic species, which can then alkylate cellular nucleophiles. It also acts as a DNA cross-linking agent [20].

The aim of this study was to measure distribution of FNP by volume and number, as well as zeta potential of particles, in aqueous solution and in RPMI 1640 with 10% FBS in dark on 37 °C during 24 h, which are basically used to treat the particles in cell culture. Based on the above mentioned fact concerning numerous biological activities of FNP, our additional goal was to test *in vitro* influence of FNP on cell's survival and activity of three antioxidative enzymes: total superoxide-dismutase (SOD), glutathione-reductase (GR) and glutathione-S-transferase (GST), in both, mitomycin C-treated CHO-K1 cells and control untreated groups of CHO-K1 cell line.

SCIENTIFIC PAPER

UDC 615:616-085:547

Hem. Ind. 69 (4) 425–431 (2015)

doi: 10.2298/HEMIND131218054S

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Paper received: 18 December, 2013

Paper accepted: 3 July, 2014

MATERIALS AND METHODS

Fullerenol nanoparticle synthesis

Fullerenol $C_{60}(OH)_{24}$ was synthesized in alkaline media by complete substitution of bromine atoms from $C_{60}Br_{24}$. Briefly, the polybromine derivative $C_{60}Br_{24}$ was synthesized through catalytical ($FeBr_3$) reaction of C_{60} in Br_2 [21]. 50 mg of $C_{60}Br_{24}$ was mixed in 5 ml of NaOH pH 10 for 2 h at room temperature. After the reaction was completed, the solvent was evaporated at 40 °C, and the mixture was rinsed five times with 10 ml portions of 80% ethanol. The aqueous solution of fullerenol (20 ml) with residual amounts of NaOH and NaBr was applied on the top of the combined ion-exchange resin DOWEX MB50 QC121815 R1 (20 g) and eluted with demineralized water until discoloration. The solution of fullerenol (pH 7) was evaporated under low pressure; a dark brown powder substance remained. Analysis: FTIR $C_{60}(OH)_{24}$: 3427, 1627, 1419, 1080 cm^{-1} ; ^{13}C -NMR (D_2O) reaction mixture: singlet peaks at δ 77.7 ppm and multiplet at δ 140 ppm, ^{13}C -NMR (D_2O) $C_{60}(OH)_{24}$: singlet peaks δ 169.47 ppm and multiplet peak δ 160–110 ppm; MALDI (matrix α -cyano-4-hydroxycinnamic acid) (m/z): 720 (C_{60}^+), 721 ($C_{60}H^+$), 722 ($C_{60}H_2^+$), 737 ($C_{60}(OH)^+$), 808 ($C_{60}(OH)_5^+$), 839 ($C_{60}(OH)_7^+$), 856 ($C_{60}(OH)_8^+$), 1009 ($C_{60}(OH)_{17}^+$), 1026 ($C_{60}(OH)_{18}^+$) and minor peak 1128 ($C_{60}(OH)_{24}^+$); DTG, DTA, TG reveal two thermal changes, in temperature of 120–395 °C, corresponding to the loss of mass of 35.7% (23.7 OH groups) and at the temperature of 430 °C loss of mass was 64.3% (this was the temperature of sublimation of C_{60}). Elementary analysis of fullerenol provided the following: C, 63.00%; H, 2.00% calc.: C, 63.83%; H, 2.13%.

Size distribution of nanoparticles

In order to obtain results for distribution of particles by volume and number, as well as zeta potential of particles, high performance analyzer Zetasizer Nano ZS (Malvern Instruments) was used. Prior to measurement, samples were tempered at 37 °C for 24 h and stored in the dark.

Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was performed with a JEOL JSM 6460 LV scanning microscope (300000× magnification). The 10 ppm aqueous solution of fullerenol was placed on a conductive carbon tape, evaporated under reduced pressure and covered with a thin gold layer about 5 nm.

Cell line treatment

All the experiments were performed on Chinese hamster ovary cell line CHO-K1, ATCC CCL61 (American Type Culture Collection Catalogue of Cell Lines and Hybridomas, 6th ed., 1988). CHO-K1 cells were cultured as a tightly flask-bonded monolayer in RPMI 1640 med-

ium (Sigma), supplemented with 10% FCS (Veterinary institute Novi Sad, NIVNS), 2 mM L-glutamine, penicillin (100 IU/ml) and streptomycin (100 μ g/ml, Galenika) at 37 °C in fully humidified atmosphere with 5% CO_2 . Single-cell suspension was obtained with 0.25% trypsin or trypsin in EDTA (Sigma). Cells were passaged twice a week in concentration of 50000–100000 cells/ml.

CHO-K1 sample cells were plated in sterile cultivation plates (Costar, 6 well) in concentration of 200000/ml and treated as follows:

Control – untreated cells

MMC – mitomycin C 0.1 μ g/ml

F1 – FNP 0.025 mg/ml

F3 – FNP 0.125 mg/ml

F1 + MMC – FNP 0.025 mg/ml 30 min before MMC 0.1 μ g/ml

F3 + MMC – FNP 0.125 mg/ml 30 min before MMC 0.1 μ g/ml.

FNP was dissolved in bi-distilled water and added in the cell culture in two final concentrations: 0.025 and 0.125 mg/ml in a 3- and 24-hour treatment.

Mitomycin C was dissolved in distilled water and added in cell culture samples in final concentration of 0.1 μ g/ml in a 3- and 24-hour treatment.

In FNP pretreated samples, FNP was added half an hour before MMC.

Dye exclusion test (DET) with Trypan blue was used to monitor the cell survival [22]. The DET test was performed by mixing 50 μ l of cell suspension with 200 μ l of 0.1% Trypan blue solution in 0.9% NaCl. After 2 min of incubation at a room temperature, the number of viable cells (unstained cells) was determined using a Burkert-Turk hemocytometer.

Survival rate was calculated according to formula:

$$\text{Survival rate (\%)} = 100 \times (\text{Total number of viable cells in experimental group} / \text{Total number of viable cells in control group})$$

All enzyme activity assays were performed in cytosolic cell fraction, in supernatant obtained by ultrasonication (Soniprep 150 MSE) (10 min at 10000 rpm at 4 °C) and kept at –80 °C. All spectrophotometric measurements were carried out in triplicates (Agilent 8453 UV/Vis spectrophotometer with thermostatted multicell position sample system).

Superoxide dismutase assay

Total (Cu–Zn and Mn) superoxide dismutase (SOD) activity measuring method was based on the ability of superoxide dismutase to inhibit the auto-oxidation of epinephrine [23]. One unit of activity was defined as the amount of enzyme necessary to decrease by 50% the rate of adrenalin auto-oxidation at pH 10.2 and 480 nm. The results were expressed as U/10⁶ of cells.

Glutathione reductase assay

Glutathione reductase (GR) was determined measuring the reduction rate of oxidized glutathione with NADPH as suitable enzyme substrate at 340 nm [24]. Activity of GR was defined as nmol of NADPH/min per 10^6 of cells.

Glutathione-S-transferase assay

Glutathione-S-transferase (GST) was based on conjugation of –SH group of reduced glutathione with 1-chloro-2,4-dinitrobenzene (CDNB) [24]. Absorbance of the conjugate CDNB–glutathione was measured at 340 nm. Activity of GST was expressed as nmol of CDNB–glutathione conjugate/min per 10^6 of cells.

Statistical analysis

The data were analyzed by Multivariate analysis of variance (MANOVA) followed by Duncan test at 0.05 significance level to compare the means using SPSS 13.0 for Windows.

RESULTS AND DISCUSSION

Results for size distribution of particles by volume (Fig. 1a) put an emphasis on inhomogeneity of the analyzed samples. In all samples can be noticed the presence of particles of dimensions within 2–30 nm, with the maximum at 5 nm. Fullerenol nanoparticles in aqueous solution are mostly within 2–8 nm, while in samples containing fetal bovine serum size varies

within 2–30 nm. Addition of FNP in the cell culture medium with 10% FBS has not influenced the size distribution of nanoparticles by volume.

Figure 1b presents nanoparticle's size distribution by number in which particles are classified into a family within 2–9 nm, with the maximum at approx. 5 nm. It can be concluded that addition of FNP in the culture medium with 10% FBS has not induced changes in the size of particles.

In Figure 2, SEM image of the fullereneol nanoparticles studied in this work shows the particles size from 30–80 nm. These findings are in accordance with DLS and AFM measurements conducted before [25].

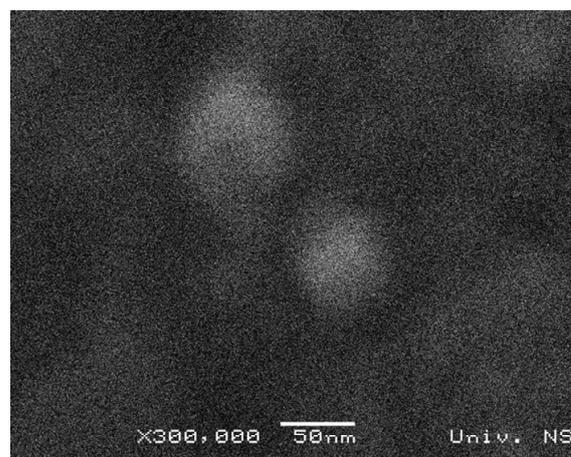


Figure 2. SEM image of film resulted from a 10 ppm aqueous solution of fullereneol.

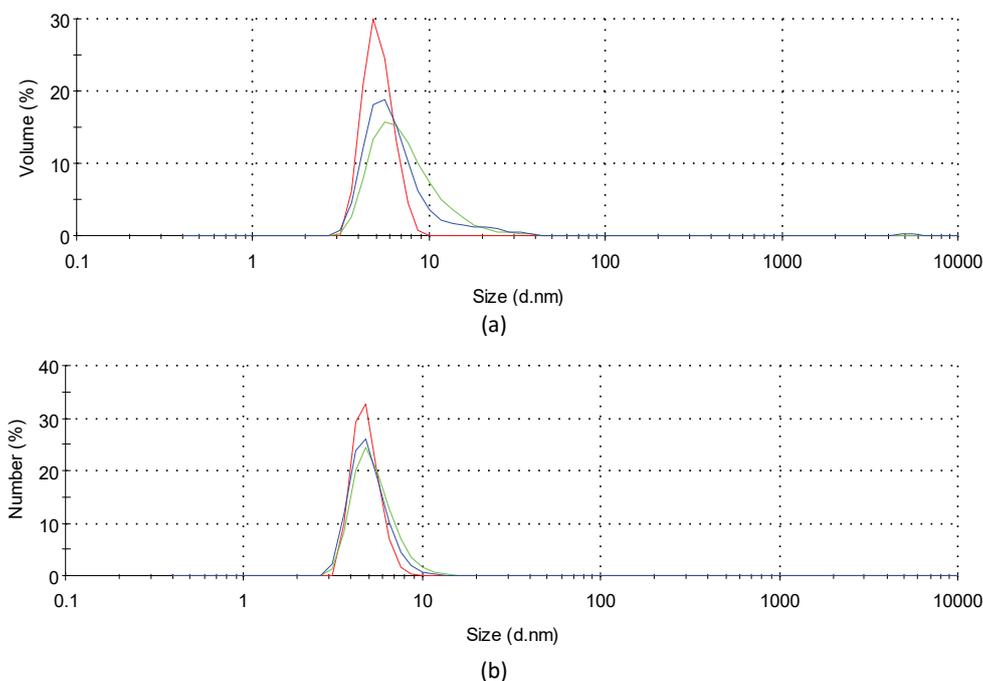


Figure 1. Size distribution of nanoparticles a) by volume and b) by number, in the following samples: cell culture medium RPMI 1640 + 10% FBS (green); aqueous solution of fullereneol (red); and fullereneol in cell culture medium RPMI 1640 + 10% FBS (blue), after incubation for 24 h at 37 °C in the dark.

Table 1 shows the results of measurements for ζ -potential of the following systems: RPMI 1640 + 10% FBS, aqueous solution of FNP pH 6, and aqueous solution of FNP in RPMI + 10% FBS (incubated for 24 h at 37 °C in the dark). ζ -potential of polyanionic fullereneol nanoparticles at pH 6 is –58 mV. After addition of FNP in the cell culture medium RPMI with 10% FBS, ζ -potential of the medium slightly changed from –3.6 to –7.9 mV; ζ -potential of nanoparticles solution changed from –58 to –7.9 mV.

Table 1. ζ -potential of the following systems: RPMI 1640 + 10% FBS, aqueous solution of FNP and aqueous solution of FNP in RPMI + 10% FBS (incubated for 24h at 37 °C in the dark)

Sample	ζ -potential, mV
RPMI 1640 + 10% FBS	–3.6
Aqueous solution of FNP: FNPaq, pH 6	–58
FNPaq + RPMI + 10% FBS	–7.9

It has been confirmed that particles' size has an important role on their adhesion to and interaction with biological cells. It is also known that the size of FNP is an important property in the toxicity analysis, since nanoparticles have a tendency to form agglomerates, which may behave differently from a single nanoparticle. Also, the presence of proteins in the culture medium can change the nanoparticles agglomeration and influence the cellular response. Nanoparticles, which are partially covered by proteins in body fluids, can change reactivity, charge and hydrophobicity [26]. It is also well-known that FNP can pass through the plasma membrane and manifest its biological effects inside the cell [27].

We can conclude that investigated FNP does not have a tendency to form agglomerates, based on the results for distribution of particles by volume and number obtained in our experiment, as well as according to the measurements for ζ -potential after incubation in cell medium. Furthermore, the presence of proteins in the culture medium does not induce significant changes in terms of nanoparticles agglomeration. Results of AFM analysis of FNP in the cell culture medium supplemented with 20% of FBS [25] revealed that FNP (which in water form aggregates of approx. 90 nm) forms a stable and homogenous solution that mostly consists of two dimer particles of 90 nm associated with one smaller nanoparticle of about 40 nm, which was assumed to be a protein from the FBS.

Results obtained in our experiment show that addition of FNP in the cell culture medium with 10% FBS does not influence the size distribution of nanoparticles and does not induce formation of such a large particle, but causes reduction in ζ -potential of nanoparticles (from –58 to –7.9 mV). Monitoring of ζ -potential is

particularly important due to possible interactions that can be favored as a result of change in particles' surface charge.

In vitro effects of FNP on the induction of cellular antioxidative capacity, actually on the increased activity of enzymes of the antioxidative system in the cells exposed to oxidative stress, are cell type- and dose-dependent [9]. Results showed that FNP did not induce genotoxic effect, on the contrary antigenotoxic effects of FNP were confirmed in the experiment done on MMC-damaged CHO-K1 cells in concentration of 11.0–221.6 μ M [8].

Our present investigation has shown that FNP in both examined concentrations moderates the activities of oxidative enzymes SOD and GR in comparison to the control, which implies that it undoubtedly enters the cells and participates in cell metabolism (Fig. 3). This mild change in enzyme activity does not affect the survival of cells.

Possible mechanisms responsible for the increased activity of SOD in CHO-K1 cells treated with FNP may be explained by the fact that fullereneol acts as NO-scavenger, which prevents superoxide consumption in the reaction of peroxynitrite anion formation, simultaneously increasing $O_2^{\cdot -}$ concentration and SOD activity as a consequence of superoxide excess [9,28].

Depending on the applied dose, the activity of enzyme GR was both, increased and reduced. The increase in activity of GR indicates the reduced cellular milieu that enables cells to efficiently scavenge free radicals, formed as a result of FNP participating in cellular metabolism, which can clarify the absence of significant influence of FNP on cell survival. Reduced activity of GR in a 3-hour experiment with FNP of higher concentration can be explained by inactivation of this enzyme due to increased level of free radicals, while results of 24-hour experiment suggest the compensatory upregulation of mRNK and reactivation of mentioned enzymes [13].

Fullereneol expressed no influence on GST activity, since it probably did not participate in detoxification of FNP. Furthermore, FNP did not induce formation of oxidative damage products to such extent to activate GST. Study conducted on the freshwater zebrafish exposed to fullereneol ($C_{60}(OH)_{18-22}(OK4)$) analyzed oxidative stress responses on fish brain. They also did not detect any statistically significant changes in GST activity or TBARS level [29].

Application of mitomycin C significantly increases the activity of all three enzymes, which presents the antioxidative response of cells to the applied agent. High activity of SOD in groups pretreated with FNP in comparison with the group treated only with MMC could be explained by the fact that fullereneol nanoparticles already act as NO-scavenger which concomi-

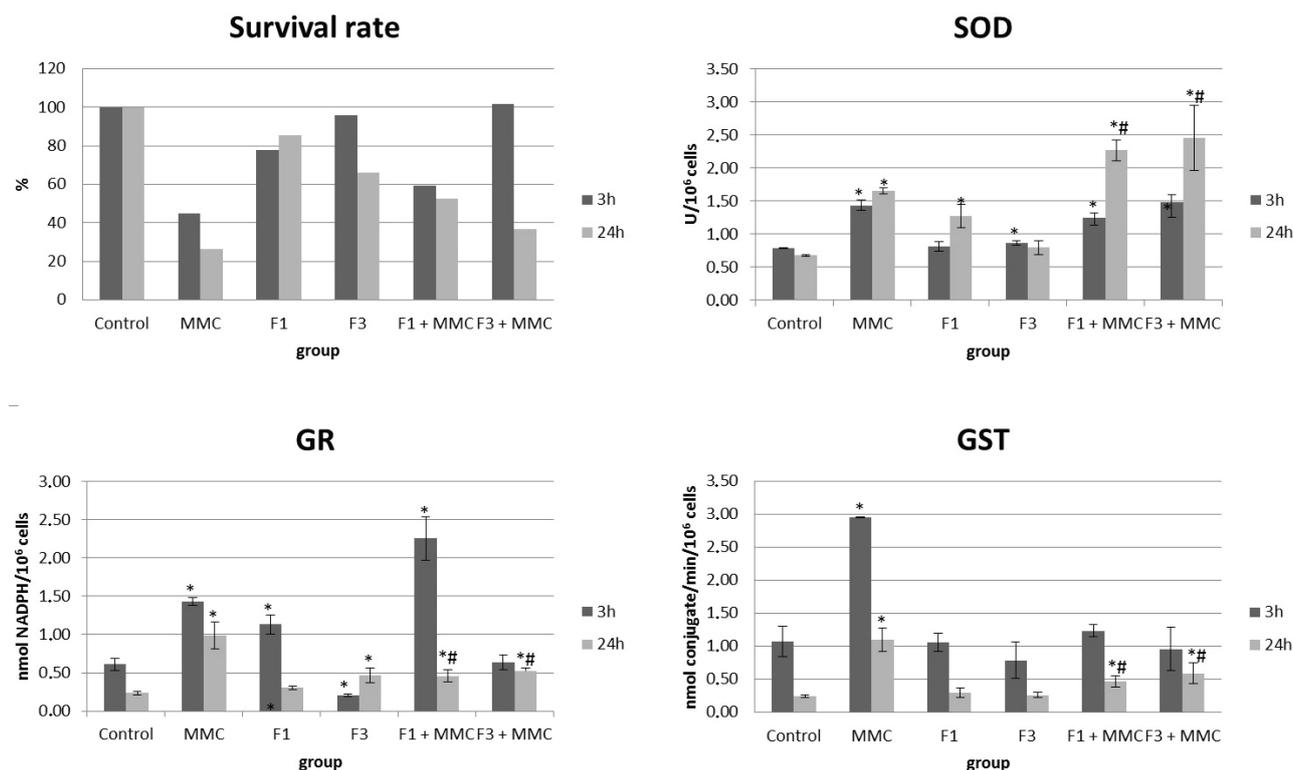


Figure 3. Effect of FNP and MMC on cells survival rate and activity of enzymes SOD, GR, GST in CHO-K1 cell line 3 and 24 h after the treatment. Control – untreated cells; MMC – mitomycin C 0.1 $\mu\text{g/ml}$; F1 – FNP 0.025 mg/ml; F3 – FNP 0.125 mg/ml; F1 + MMC – FNP 0.025 mg/ml 30 min before mitomycin C 0.1 $\mu\text{g/ml}$; F3 + MMC – FNP 0.125 mg/ml 30 min before mitomycin C 0.1 $\mu\text{g/ml}$; * – $p < 0.05$ vs. control group; # – $p < 0.05$ vs. group treated only with mitomycin C.

tantly increases the O_2^- concentration and SOD activity. Addition of MMC leads to more superoxide production and additional influence on the balance between NO and O_2^- in cells, and consequently high SOD activity.

Pretreatment with the lower concentration of FNP notably increases the activity of GR even in comparison to mitomycin C. This may happen because FNP in such a low concentration does not exhibit potent antioxidative potential, where 24 h after pretreatment the activity of this enzyme was yet to reach the control level, but still was statistically significantly lower than the values in MMC groups. Survival rate of pretreated cells was significantly higher in comparison to those treated only with MMC, which confirms the protective properties of FNP in the range of used concentrations in MMC-damaged CHO-K1 cells [8].

In acute phase, the higher concentration of FNP completely neutralizes the consequences of MMC treatment and normalizes the activity of GR, which was reflected also on the survival percentage of the cells (101.74%). Although the activity of GR after 24 h was increased in comparison to control, the survival rate was significantly higher than survival rate in MMC treated group. The results of GST activity also point out the protective effects of FNP since pretreatment with FNP significantly neutralizes the influence of MMC on the activity of this enzyme. These findings are in

accordance with the study conducted A549 cells where pretreatment with $\text{C}_{60}(\text{OH})_{24}$ attenuated hydrogen peroxide-induced apoptotic cell death by induction of phase II detoxifying enzymes [30].

As previous works concluded, polyhydroxylated fullerenes, as strong antioxidants, influence the cellular redox state and thus could reduce cell proliferation, which could be exploited for the use of fullereneol as a cytoprotective agent [4,31,32].

CONCLUSION

In summary, the present results demonstrate that polyanion fullereneol nanoparticles reduce mitomycin C-induced oxidative stress in dose- and time-dependent manner, and therefore possess beneficial effects on preventing drug toxicity in CHO-K1 cells. Applied alone, fullereneol nanoparticles influence the cell metabolism moderately and not in such a manner to induce any severe irreversible changes that would consequently lead to the cell death.

Acknowledgment

This work is supported by The Ministry of Education, Science and Technological Development of the Republic of Serbia, project No. III45005 and the Provincial Secretariat for Science and Technological

Development of Vojvodina (grant number 114-451-2056/2011-01).

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IZVOD

RASPODELA FULLERENOLSKIH NANOČESTICA PO VELIČINI U ĆELIJSKOM MEDIJUMU I NJIHOV UTICAJ NA ANTIOKSIDATIVNE ENZIME U OVARIJALNIM ĆELIJAMA KINESKOG HRČKA

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Zahvaljujući širokoj biološkoj aktivnosti, npr. citoprotektivnom i antioksidativnom svojstvu, fullereneol ima značajnu ulogu u biomedicinskim istraživanjima, na šta ukazuju i rezultati brojnih istraživanja. Cilj ovog istraživanja je bio merenje zeta potencijala i raspodele fullereneol nanočestica u medijumu RPMI 1640 + 10% FBS (Fetal Bovine Serum), kao i ispitivanje uticaja fullereneola na preživljavanje ćelija i aktivnost tri antioksidativna enzima: superoksid-dismutaze, glutation-reduktaze i glutation-S-transferaze, u mitomicinom C tretiranoj ćelijskoj liniji. Istraživanje je obavljeno na ćelijama jajnika kineskog hrčka CHO-K1 (*Chinese Hamster Ovary cells*) koristeći DET test (*Due Exclusion Test*) za brojanje ćelija, kao i set spektrofotometrijskih metoda za određivanje antioksidativne aktivnosti. Ćelije su pre tretmana mitomicinom C tretirane fullereneolom u dve različite koncentracije, a potom inkubirane i analizirane nakon 3 i 24 h. Dodavanje fullereneolskih nanočestica u medijum sa 10% FBS nije izazvalo promene u raspodeli veličina čestica po broju ili zapremini, dok se vrednost zeta potencijala medijuma (ζ) promenila sa $-3,6$ na $-7,9$ mV. Fullereneol ispoljava protektivni efekat na ćelije CHO-K1 koje su tretirane mitomicinom C. Mitomicin C povećava aktivnost sva tri ispitana enzima, dok sam fullereneol u veoma maloj meri utiče na aktivnost pomenutih enzima. Pretretman sa fullereneolom smanjuje stres indukovano mitomicinom C po vremenski i dozno zavisnom obrascu. Naše istraživanje potvrđuje da nanočestice fullereneola utiču na redoks stanje i enzimsku aktivnost ispitivane ćelijske linije, što ukazuje na to da mogu sniziti nivo ćelijske proliferacije i naći primenu kao citoprotektivni agensi.

Ključne reči: Fullereneol • Mitomicin C • Antioksidativni enzimi • CHO K1 ćelijska linija