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EFFECTS OF FULLERENOL NANO PARTICLES $C_{60}(OH)_{24}$ ON MICRONUCLEI AND CHROMOSOMAL ABERRATIONS' FREQUENCY IN PERIPHERAL BLOOD LYMPHOCYTES

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Fullerenol $C_{60}(OH)_{24}$ nano particles (FNP) show various biological activities in different experimental models. We evaluated genotoxic and antigenotoxic effects of FNP on human peripheral blood lymphocytes (PBL) using two cytogenetic assays. The effects of FNP were evaluated on the frequency of chromosomal aberrations (CA) and micronuclei (MN) on undamaged and mitomycin C (MMC)-damaged PBL.

FNP number distribution in a culture medium with serum showed that predominant particles were about 180 nm and 90 nm respectively. Cytogenetic assays showed that FNP decreased chromosomal aberrations and micronucleus frequency on the undamaged and the MMC-damaged human PBL at concentration range from 5.54 μM to 221.60 μM. Our research confirmed that FNP did not exhibit genotoxic but induced antigenotoxic effects at subcytotoxic concentrations on human lymphocytes.

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Keywords: Fullerenol, nano particles, chromosomal aberration, micronuclei, genotoxicity, antigenotoxicity

Abbreviations: FNP – fullerenol $C_{60}(OH)_{24}$ nano particles, PBL – peripheral blood lymphocytes, CA – chromosomal aberration, MN – micronuclei, MMC – mitomycin C, AFM – atomic force microscopy, ROS – reactive oxygen species, HOPG – highly orientated pyrolytic graphite, PHA – phytohemagglutinin, CBMN – cytokinesis-block micronucleus assay, DET – dye exclusion test, DLS – Dynamic Light Scattering, CytB – Cytochalasin-B, FBS – Fetal Bovine serum, PBS – phosphate buffer, NDI – nuclear division index, BNC – binucleated cells, MonoNC – mononuclear cells, MNC – multinuclear cells, Dic – dicentric chromosome, Ac – acentric fragment, NP – nano particle

1. Introduction

Polyhydroxylated derivatives of fullerene C_{60}, fullerenols ($C_{60}(OH)_x$), along carboxyfullerene and other polar cycloaddition products, have a significant place in a recent biological research [1, 2]. The best-known biological characteristic of fullerenols is their antioxidative property [3, 4, 5]. Fullerenols show strong antioxidative activity against ROS,

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induced by ionizing radiation [6, 7, 8], CCl_4 [3] or doxorubicin [2, 9, 10, 11] in different biological models. Nano particles of $C_{60}(OH)_{24}$ are the mitochondrial protective antioxidants with direct radical scavenging activity and indirect antioxidative activity [5]. Other biological activities of FNP comprise antiproliferative [12, 13] and neuroprotective effects [14, 15]. Jiao et al. [16] showed that fullerenol decreases expression of genes responsible for angiogenesis in metastatic tumors. However, FNPs at higher concentrations in the presence of UV or polychromatic light sources are able to produce ROS, which emphasizes their possible use as pro-oxidants [17, 18].

Genotoxicity of nano particles may result from direct effects of nano particles or their ROS products on DNA, or through indirect effects mediated by the ROS, induced in inflammatory processes or through dysregulation of the DNA repair enzymes. Several studies have shown that pristine C_{60} and some derivatives may cause the DNA damage [19, 20, 21, 22], but others revealed no genotoxicity of fullerenes [23, 24, 25, 26, 27, 28].

Obtained contradictory data are mostly due to differences in the study design, fullerene type and the preparation, exposure time, cell type, the experimental model and the endpoint measured. Generally, it is assumed that fullerenes do not have direct effect on the DNA [23]. Because of potential FNP usage in biomedicine, it is necessary to examine how FNP affects the undamaged DNA and how it works on the DNA damaged by a known mutagen.

Our previous results demonstrated that fullerenol $C_{60}(OH)_{24}$ nano particles do not induce genotoxic effects in wide range of doses (11–221μM), but protect both undamaged and mitomycin C-damaged Chinese hamster ovary cells, CHO-K1 cells [29]. Since, currently, there are little data available on the genotoxic activities of FNP on human cells and importance of physicochemical characteristics of FNP, we evaluated fullerenol genotoxicity in the set of experiments: characterization of stable FNP in water and in the cell culture medium with Fetal Bovine serum (FBS) (i), determination of FNP cytotoxicity on human peripheral blood lymphocytes (PBL) at a range of concentrations (ii), and *in vitro* micronucleus test and chromosomal aberration test on undamaged and mitomycin C-damaged human PBL (iii).

2. Material and methods

CHEMICALS

SYNTHESIS. Fullerenol $C_{60}(OH)_{24}$ was synthesized in alkaline media by complete substitution of bromine atoms from $C_{60}Br_{24}$ [30]. 80 mg of $C_{60}Br_{24}$ was mixed in 40 cm^3 of NaOH, pH=10.5, for 4 hours at 23^0C. After the reaction was completed, the solvent was evaporated at 60^0C, and the mixture was 12 times rinsed with 20 cm^3 portion of 80% ethanol with sonication. Water solution (90 ml) of fullerenol with residual amounts of NaOH and NaBr was applied to the top of the combined ion exchange resin (40g DOWEX MB50 QC121815 R1) and eluted with demineralized water until discoloration. Water solution of fullerenol was evaporated under low pressure; a dark brown amorphous powder substance remained. *Analysis*: FTIR $C_{60}(OH)_{24}$: 3427, 1627, 1419, 1080 cm^{-1}; ^{13}C NMR (D_2O) $C_{60}(OH)_{24}$: singlet peaks δ=169.47 ppm and multiplet peak 160-110 ppm; MALDI MS (*m/z*): 720 (C_{60}^+), 993 ($C_{60}(OH)_{16}^+$), 1043 ($C_{60}(OH)_{19}^+$), 1061 ($C_{60}(OH)_{20}^+$), 1128 ($C_{60}(OH)_{24}^+$); DTG, DTA, TG: reveals two thermal changes in temperature of 120–395^0C, corresponding to the loss of mass of 35.7% (23.7 OH groups) and at the temperature of 430^0C the loss of mass was 64.3% (this was the temperature of sublimation of C_{60}). Elementary analysis: C-63.0%, H-2.0 % calc.: C-63.83%, H-2.13%.

VISUALIZATION. Morphology and structure evaluation of: (*i)* water solution $C_{60}(OH)_{24}$ and (*ii)* $C_{60}(OH)_{24}$ in RPMI 1640 +20% FBS was performed using the Atomic Force Microscopy (AFM). Surface topography and phase images were simultaneously acquired by standard AFM tapping mode using a commercial NanoScience-Team Nanotec GmbH SNC (Solid Nitride Cone) AFM probe, with the tip radius lower than 10 nm. Highly orientated pyrolytic graphite HOPG was used as surface. Multimode quadrex SPM with Nanoscope IIIe controller (Veeco Instruments, Inc.), operated under ambient conditions was used.

Fullerenol powder was dissolved in demineralized water at concentration of 3 mg/ml ("stock 1" solution) and then diluted with RPMI 1640 medium (Sigma) to set a "stock 2" solution

with concentration of 0.6mg/ml as a "working" solutions which was used for majority of the examined endpoints.

The alkylating agent mitomycin C (MMC, CASRN 50-07-7, Bristol-Myers Squibb, USA), as a well-known inducer of sister chromatid exchanges, structural chromosomal aberrations and micronuclei, was used as a positive control.

MMC was dissolved in a phosphate buffer (PBS). Cytochalasin B (CASRN 14930-96-2) was from Sigma and Colcemid solution (CASRN 477-30-5) and Phytohemagglutinin (PHA) were from PAA Laboratories (GmbH). RPMI 1640 medium and Trypan blue solution were from Sigma (USA), penicillin and streptomycin were from ICN (USA) and Fetal Bovine serum was from PAA Laboratories (GmbH).

MMC was added at different final concentrations, defined in our previous pilot experiments as follows: we used five concentrations of MMC (0.15, 0.3, 0.6, 0.9 and 1.5 nM), for G0, G1/S and G1/S/G2/M phase cell cycle in both CBMN and CA assays (data not shown).

Cell culture

The peripheral blood sample was obtained from a 33-year-old healthy female, not having been exposed to any chemical or physical agent during the last 6 months. Venous heparinized blood (0.45 ml) was added to 5 ml of RPMI 1640 medium containing 20% FBS for chromosomal aberrations or 15% FBS for micronucleus assay, 2% phytohemagglutinin and antibiotics (100 IU/mL penicillin, and 100µg/mL streptomycin). Blood culture was incubated at 37^{o}C in a humidified atmosphere with 5% of CO_2. The cultures were set up in triplicate vessels per concentration for each experimental treatment condition.

Cell survival and cytotoxicity test

FNP solution was added to cell culture in final concentrations of 8.86, 44.3, 88.6, 221.6 and 443.2 µM (0.01, 0.05, 0.1, 0.25 and 0.5 mg/ml). The FNP treatment on human lymphocytes was identical for both MN and CA tests. Incubation periods were 24 h for G0 and G1/S phases of the cell cycle and 4h for G1/S/G2/M phases of the cell cycle.

Dye exclusion test (DET) with Trypan blue [31] was used to monitor the cell survival and the FNP cytotoxicity. The DET test was performed by mixing 50 µL of cell suspension with 200 µL of 0.1% Trypan blue solution in 0.9% NaCl. After 2 minutes of incubation at a room temperature, the number of viable cells (unstained cells) was determined using a Burker-Turk hemocytometer. Cell survival and cytotoxicity percentage were determined from two independent experiments done in triplicate for each FNP concentration according to the following formulas:

Percentage of viable cells = (number of viable cells / total cell number) x 100

Cytotoxicity percentage = [1- (number of viable cells / total cell number)] x 100

LC_{50} is the FNP concentration that induces 50% of cell deaths.

Based on the LC_{50} values, we selected three subcytotoxic concentrations of fullerenol as 50%, 25% and 12.5% of LC_{50} values for all examined genotoxic endpoints.

Micronucleus assay

Lymphocyte cultures were set up according to recommendations for testing of the genotoxic effects of compounds [32]. These recommendations refer to the cell cycle phases (G0, G1/S and G1/S/G2/M), as well as to the duration of treatment of investigated compound in a culture of human lymphocytes.

For G0 phase of the cell cycle, the cultures were incubated for 24h, treatment with FNP and /or MMC in different concentrations (FNP: 11.08, 22.16 and 44.3µM; MMC: 1.5nM). Thereafter, the medium was replaced with a fresh one containing PHA. For the G1/S phase of the cell cycle, 20h after the PHA stimulation, there was 24h treatment with FNP and /or MMC in different concentrations (FNP: 27.65, 55.4 and 110.8µM; MMC: 1.5nM) and thereafter the medium was replaced with a fresh one. For the G1/S/G2/M phase of the cell cycle, a 4-hour-treatment with FNP and/or MMC at different concentrations (FNP: 27.65, 55.4 and 110.8 µM;

MMC: 1.5nM), 44h after the PHA stimulation, and the medium was replaced with fresh one. In the study of genotoxicity, the controls were represented by PBL cultures without FNP treatment, while in the study of antigenotoxicity, they were represented by PBL treated with MMC. Cytochalasin-B (CytB) was added to the lymphocyte cultures at a final concentration of 6 μg/mL in the 44th h for all of three experimental sets. Harvesting was in the 72nd h according to the published procedures [33] with minor modifications regarding staining procedures. The cells were washed in a fresh medium, briefly exposed to a cold hypotonic solution (0.56% KCl) and fixed three times with methanol: glacial acetic acid (3:1,v/v). Air-dried slides were stained with Giemsa 2% in distilled water for 9 min. CBMN assay was performed, analyzing more than 1000 cells per each sample. Standard criteria were used for the identification of micronuclei [32]. Monitored values included number of mono-nucleated, bi-nucleated and multi-nucleated cells, incidence of micronuclei and nuclear division index (NDI). Micronucleus incidence was presented as a number of micronuclei per 1000 examined binuclear cells.

NDI was calculated according to the formula:

NDI = (M1 + 2M2 + 3(M3+M4)) / N, where M1-M4 represents the number of cells with 1 to 4 nuclei found, respectively, and N is the total number of scored cells [33]. The NDI is a measure of the average number of cell cycles that a cell population passes through, considering both three-nucleated and tetra nucleated cells in the same category.

Chromosome aberration assay

The chromosome aberration assay was performed according to the standard procedures [34].

For G0 phase cell cycles, the cultures were incubated for 24h, treatment with fullerenol and /or MMC in different concentrations (FNP: 5.54, 11.08 and 22.16μM; MMC: 1.5 nm) and after that, the incubation medium was replaced with a fresh one containing PHA. For the G1/S phase cell cycles, a 24h-treatment with FNP and /or MMC in different concentrations (FNP: 11.08, 22.16 and 44.3 μM; MMC: 1.5nM) was initiated in the 20th h after the PHA stimulation and after the treatments, the medium was replaced with a fresh one. For the G1/S/G2/M phase cell cycles, a 4h-treatment with fullerenol and/or MMC in different concentrations (FNP: 55.4, 110.8 and 221.6μM; MMC: 1.5nM) was initiated in the 44th h after the PHA stimulation, and then the medium was replaced with a fresh one. In the study of genotoxicity, the controls were represented by PBL cultures without FNP treatment and in the study of antigenotoxicity by PBL cultures with MMC. Colcemid solution was added in the 48th h, in a final concentration of 0.1 μg/mL, in order to capture all cells undergoing their first nuclear division. The cells were harvested 1 h later. The cells were treated with hypotonic solution (0.56% KCl) for 25 min at 37°C in fully humidified atmosphere with 5% of CO_2 and fixed three times with methanol: glacial acetic acid (3:1, v/v) for 15 min. After the storage at 2-8°C overnight, a suspension of cells in fresh fixative was dropped onto cold glass microscope slides and air-dried. The slides were stained with Giemsa 2% in distilled water for 9 min and scored for structural chromosome aberrations.

The analysis of chromosome aberrations was performed by the analysis of a minimum of 100 metaphase cells per point. The CA was determined only in the metaphases containing 46 chromosomes. Structural CA was categorized as a chromatid-type and chromosome-type, according to Savage [35].

The prepared material was observed and analyzed by light microscopy (Olympus BX51). The images were captured by a 3.2-mega pixel digital camera (Olympus CAMEDIA C3040 Zoom) connected to the computer.

Statistical Analysis

Results are presented as the mean values ± SD obtained from one experiment done in triplicate for each fullerenol concentration. The differences between the control and examined fullerenol concentration were analyzed by one way ANOVA with Fisher-LSD using STATISTICA Release 8, to find significant differences in the frequency of MN and CA. Statistical decisions were based on a significance level of at least 0.05 and 0.005.

3. Results

AFM study of FNP in water and cell culture medium

AFM images of the FNP water solution and the complete culture medium (RPMI 1640 + 20% FBS) after 24h incubation at 37^0C are presented in Figures 1 and 2.

Water solution of FNP was inhomogeneous and most of the nano particles were retained on HOPG terraces (Figure 1a.). Retention of FNP on HOPG terraces indicates hydrophilic property of the particles. Grouping of particles in bigger aggregates on the HOPG terraces do not reflect real image of the FNP water solution. Figures 1b and 1d show FNP of similar shape and size (86.9, 78.2, 82.6, 86.9, 86.9, 78.2, 86.9 nm), which was confirmed by measurements presented in Figure 1c. Dynamic Light Scattering (DLS) measurements of the FNP number distribution in water were confirmed by the AFM analysis. Nanoparticle size ranged between 50 nm and 130 nm and the most abundant were particles of 90 nm. Particles larger than 250 nm were not present in a significant percent. Heating of the FNP water solution at 37^0C during 24h did not induce significant changes in particle number distribution when compared with a control measurement at 23^0C.

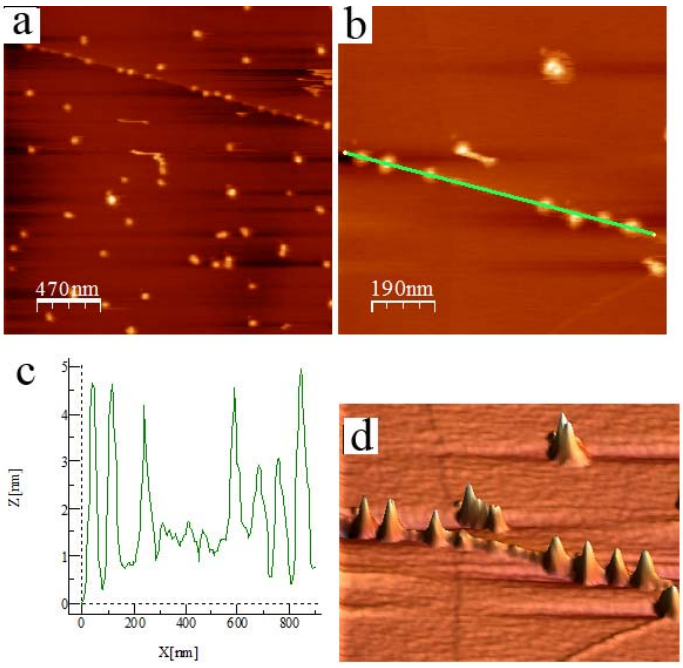

Fig. 1. AFM images of water solution of FNP after 24h at 37^0C
(a) Large-scale image, 2400 x 2400 nm^2, and (b) small-scale image, 960 x 960 nm^2, of nano particles of about 87 nm on HOPG surface; (c) corresponding cross-section of fullerenol nano particles. Maximal peak of the particle is 4.9 nm; smaller particle peaks are 2.9 nm and 1.5 nm, respectively; (d) 3D image of FNP on the HOPG surface

Fig. 2a and 2b show nanoaggregates of 180 nm composed of two fullerenol particles. Each particle is approximately 90 nm. Each aggregate contains also the third nanoparticle of about 40nm. Based on shape and size, we assumed that the third nanoparticle might be a protein from the FBS. The DLS measurements of FNP number distribution showed that predominant particles were between 185nm and 93.40nm, respectively. AFM and DLS analyses of RPMI 1640+20% FBS reveal particles from 29nm to 61nm (data not shown). Heating of the FNP solution in RPMI 1640 with 15% FBS or 20% FBS at 37^0C during 24h, did not induce significant changes in particle shape or particle number distribution. FNP in RPMI 1640 (regardless the FBS percentage) forms homogeneous system with a dominant presence of 180nm nanoaggregates.

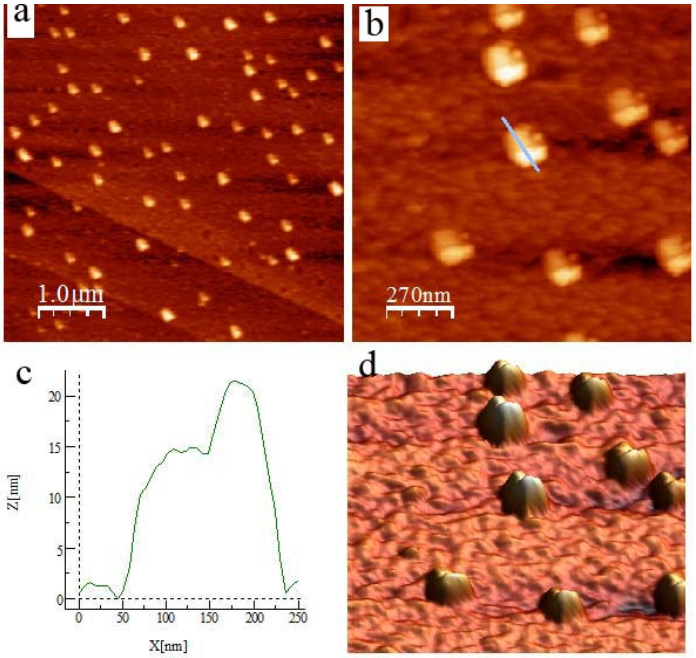

Fig. 2. AFM images of solution FNP in RPMI 1640 + 20% FBS after 24h at 37^0C (a) Large-scale image, 5000 x 5000nm^2, and (b) small-scale image, 1400 x 1400nm^2, of nano particles of about 180nm on HOPG surface; (c) corresponding cross section of fullerenol nano particles. Maximal peak of the particle is 21.4nm; smaller particle peak is 14.9nm; (d) 3D image of FNP in RPMI 1640 + 20% FBS on the HOPG surface

Cell survival and LC_{50} for FNP on undamaged PBL

FNP induced concentration and time-dependent cell death in peripheral blood lymphocytes (Figure 3 and 4). With increasing FNP concentrations, the cell survival decreased in all phases of the cell cycles under the conditions corresponding to the CBMN test.

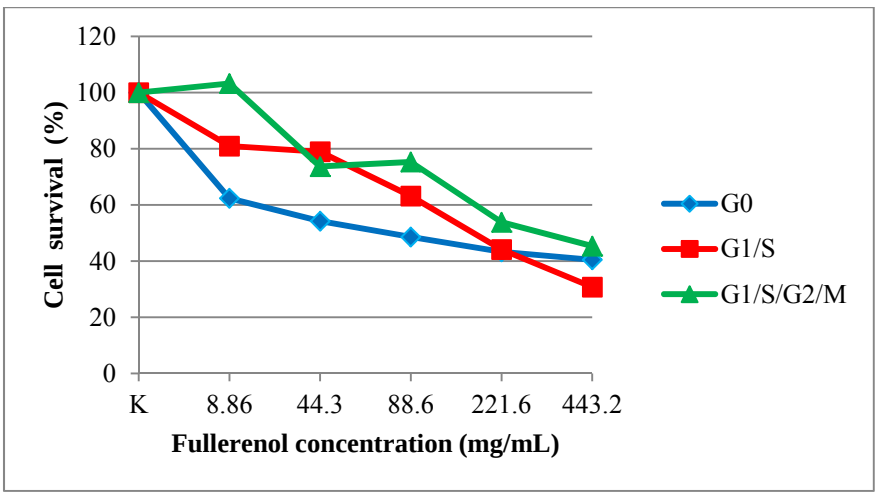

Fig. 3. The influence of FNP on cell survival in conditions corresponding to the CBMN test

Under the conditions corresponding to the CA test, with increasing FNP concentrations, the cell survival also decreased in all phases of the cell cycles (Figure 4).

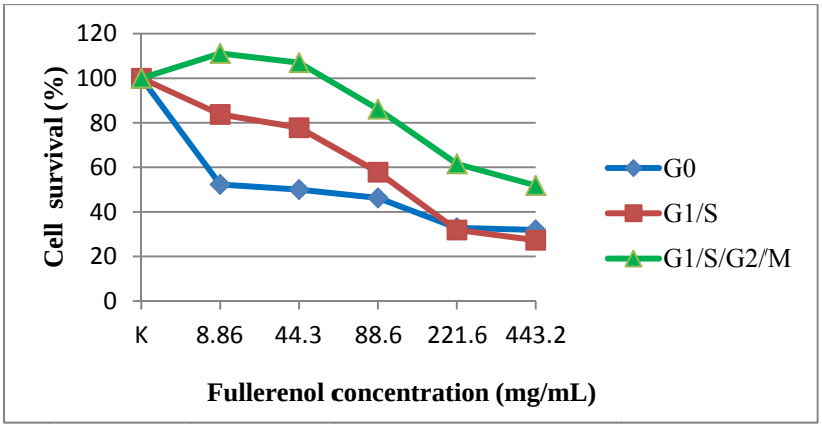

Fig. 4. The influence of FNP on cell survival in conditions corresponding to the CA test

The LC_{50} values of FNP in the CBMN and the CA tests were in a range of micromolar concentrations but different due to methodological design of the two tests (Table 1). Similar values were obtained in our previous experiments on CHO-K1 cells [29].

Table 1 LC_{50} values of FNP for CBMN and CA assay

Cell cycles phases	Assay	
	CBMN	**CA**
G0	88.6	44.3
G1/S	221.6	88.6
G1/S/G2/M	221.6	443

LC_{50} values are expressed in µM

Effects of FNP on micronucleus frequency

Fullerenol nanoparticles induced a decrease of micronucleus frequency in all phases of the cell cycles of human lymphocytes compared to control i.e. FNP-untreated PBL. Moreover, MN frequency significantly decreased at higher FNP concentrations (Figure 5).

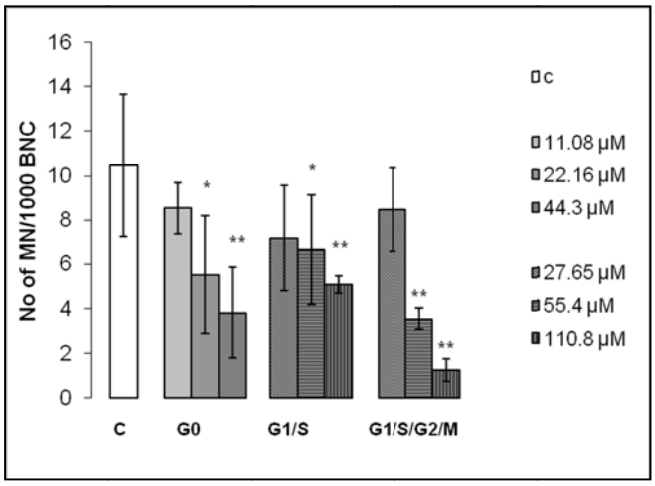

Fig. 5. Induction of micronuclei by FNP in PBL
*C-control, * Significantly different from the control (ANOVA, Fisher-LSD test - $p < 0.05$),*
** * Significantly different from the control (ANOVA, Fisher-LSD test - $p < 0.005$)*

In MMC-damaged human lymphocytes, FNP significantly decreased MN frequency in all phases of the cell cycle compared to control i.e. FNP-untreated human lymphocytes (Figure 6).

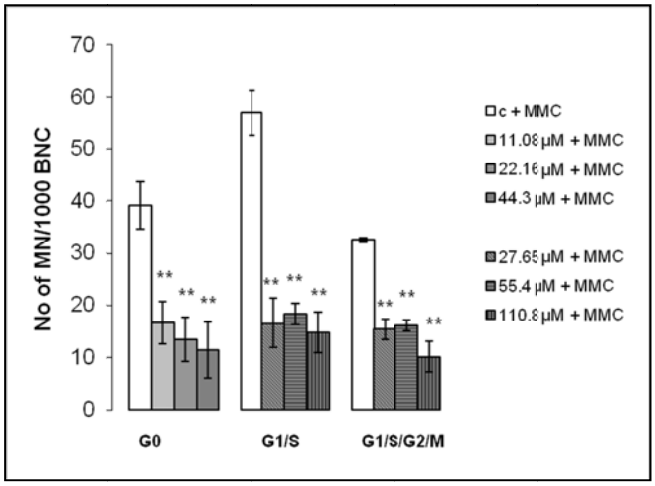

Fig. 6. Induction of micronuclei by FNP on MMC-damaged PBL
*C-control, * * Significantly different from the control (ANOVA, Fisher-LSD - $p < 0.005$)*

Effects of FNP on nuclear division index

FNP increased nuclear division index (NDI) of human lymphocytes at a range of concentrations (11.08-110.8 µM) (Table 2). However, in MMC-damaged lymphocytes, the NDI values were decreased in all phases of the cell cycle. After the FNP treatment on MMC-damaged cells, with the increase of the FNP concentrations, the NDI values slightly increased, but always remained below the control values.

Table 2. NDI values in treatment with FNP and FNP/MMC

Treatment Period	Fullerenol (µM)	NDI	Treatment Period	Fullerenol (µM) + MMC (nM)[a]	NDI
Control	0	1.85	**G0**	0	1.68
G0	11.08	1.89		11.08	1.64
	22.16	1.87		22.16	1.65
	44.3	1.91		44.3	1.68
G1/S	27.65	1.86	**G1/S**	0	1.76
	55.4	1.91		27.65	1.61
	110.8	1.88		55.4	1.63
G1/S/G2/M	27.65	1.84	**G1/S/G2/M**	110.8	1.64
	55.4	1.84		0	1.75
	110.8	1.87		27.65	1.66
				55.4	1.67
				110.8	1.68

MMC[a]- 1.5nM of mitomycin C

Effects of FNP on chromosome aberrations frequency

Fullerenol nanoparticles significantly decreased CA frequency at concentration range from 5.54 µM to 221.6 µM in all phases of the PBL cell cycle compared to the control value (Figure 7).

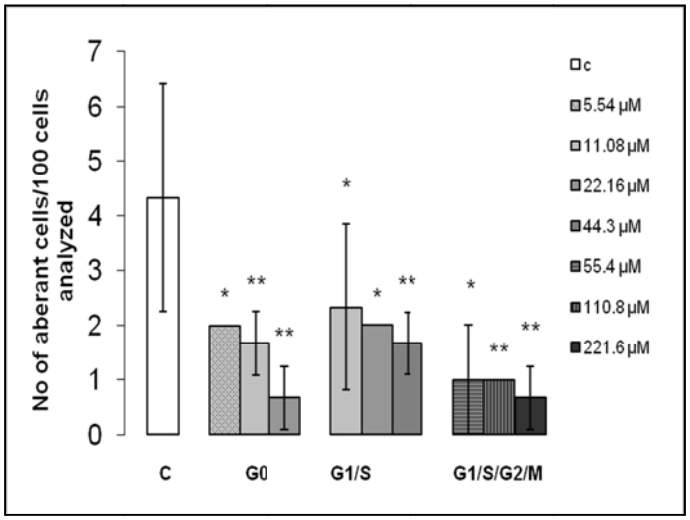

Fig. 7. Induction of chromosome aberrations by FNP in PBL
*C-control, * Significantly different from the control (ANOVA, Fisher-LSD test - p < 0.05),*
** * Significantly different from the control (ANOVA, Fisher-LSD test - p < 0.005)*

Despite the reduction in a CA frequency in the FNP treatment of human lymphocytes, regarding the type of the chromosomal aberrations, a chromatid type of alterations dominated over chromosome in all phases of the cell cycle (Table 3).

Table 3. Types of chromosome alterations obtained in treatment of human lymphocytes with FNP

Treatment Period	Fullerenol (µM)	Types of chromosome alterations								
		Chromatid			Chromosome					
		Gap	Break	Total no. of alterations	Gap	Break	Dic	Ring	Ac	Total no. of alterations
Control	0	1.33	2.33	3.66	0.00	0.67	0.00	0.00	0.00	0.67
G0	5.54	1.33	0.33	1.66	0.33	0.00	0.00	0.00	0.00	0.33
	11.08	0.67	1.00	1.67	0.00	0.00	0.00	0.00	0.00	0.00
	22.16	0.00	0.67	0.67	0.00	0.00	0.00	0.00	0.00	0.00
G1/S	11.08	0.67	0.67	1.34	0.67	0.33	0.00	0.00	0.00	1.00
	22.16	1.00	0.67	1.67	0.00	0.67	0.00	0.00	0.00	0.67
	44.30	1.00	0.00	1.00	0.33	0.33	0.00	0.00	0.00	0.66
G1/S/G2/M	55.40	0.67	0.33	1.00	0.00	0.00	0.00	0.00	0.00	0.00
	110.80	0.67	0.00	0.67	0.33	0.00	0.00	0.00	0.00	0.33
	221.60	0.33	0.33	0.66	0.00	0.00	0.00	0.00	0.00	0.00

Three hundred cells were scored for each concentration of fullerenol: the data are presented as % of chromosome alterations.

FNP treatment of MMC-damaged human lymphocytes significantly decreased a CA frequency in all phases of the cell cycle compared to the control values (Figure 8).

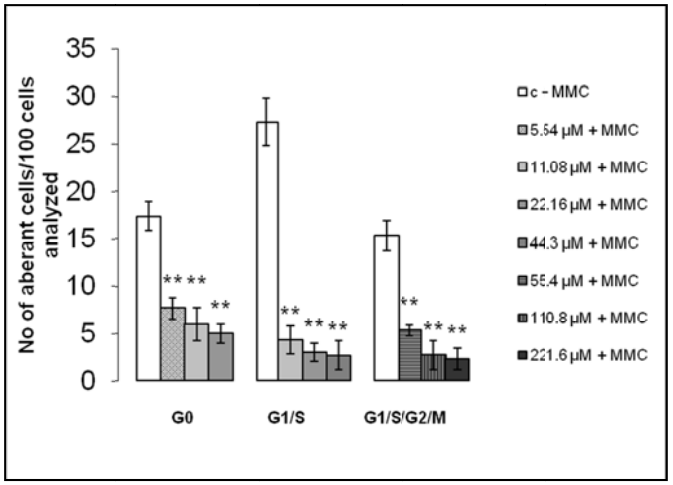

Fig. 8. Induction of chromosome aberrations by FNP on MMC-damaged PBL
*C-control, * Significantly different from the control (ANOVA, Fisher-LSD test - $p < 0.05$),*
** * Significantly different from the control (ANOVA, Fisher-LSD test - $p < 0.005$)*

Mitomycin C induced mainly chromosome type of alterations but FNP on MMC-damaged human lymphocytes induced mainly chromatid type of alterations in all phases of the cell cycles (Table 4).

Table 4. Types of chromosome alterations obtained after the FNP treatment of MMC-damaged PBL

Treatment Period	**Fullerenol (µM) + MMC[a]**	**Types of chromosome alterations**								
		Chromatid			**Chromosome**					
		Gap	Break	Total no. of alterations	Gap	Break	Dic	Ring	Ac	Total no. of alterations
G0	0	4.67	1.67	6.34	7.00	1.67	0.67	0.00	2.00	11.34
	5.54	1.67	2.00	3.67	0.67	2.33	0.00	0.00	1.00	4.00
	11.08	1.67	1.67	3.34	0.33	1.00	0.00	0.00	2.33	3.66
	22.16	2.67	1.67	4.34	0.33	0.33	0.00	0.00	0.00	0.66
G1/S	0	2.33	1.33	3.66	3.00	3.00	3.00	0.00	13.33	22.33
	11.08	1.00	1.33	2.33	1.00	0.33	0.00	0.00	0.67	2.00
	22.16	1.33	0.67	2.00	0.00	0.67	0.00	0.00	0.33	1.00
	44.30	1.00	0.67	1.67	1.00	0.00	0.33	0.00	0.00	1.33
G1/S/G2/M	0	3.33	0.67	4.00	2.33	5.00	1.67	0.00	2.67	11.67
	55.40	2.33	1.00	3.33	0.33	0.67	0.00	0.00	1.00	2.00
	110.80	1.33	1.00	2.33	0.00	0.33	0.00	0.00	0.00	0.33
	221.60	2.00	0.00	2.00	0.00	0.00	0.00	0.00	0.00	0.00

MMC [a]-1.5nm of mitomycin C, three hundred cells were scored for each concentration of fullerenol: the data are presented as % of chromosome alterations

4. Discussion

Results of our previous study showed that fullerenol nanoparticles were not genotoxic at subcytotoxic concentrations for Chinese hamster ovarian cell line CHO-K1 [29]. However, literature data about genotoxicity of FNPs on mammalian experimental models are still insufficient. Considering a great potential of FNPs usage in biomedicine, our aim was to

investigate genotoxic and antigenotoxic potential of FNPs on human chromosomes. Therefore, we examined two different cytogenetic endpoints in human peripheral blood lymphocytes cultivated *in vitro* in the presence of FNP. Our results showed that FNP at subcytotoxic concentrations decreased the MN and the CA values on undamaged and on MMC-damaged human lymphocytes in all phases of the cell cycle.

Because physical and chemical characteristics of nanoparticles such as size, shape or charge may influence both types of genotoxicity (primary and secondary) [36], we examined these characteristics using AFM microscopy, Z potential and number of distribution of nanoparticles.

It is 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 significantly change the NPs agglomeration and influence the cellular response. It is known that nanoparticles, which are partially covered by proteins in body fluids, can change reactivity, charge and hydrophobicity [37]. Since the endpoints such as micronuclei and chromosomal aberrations reveal the potential of nanoparticles to induce genotoxicity and that FNP size and protein presence can affect genotoxicity, we performed AFM analysis of FNP solution in water and in the complete cell culture medium, which was used through the assays. AFM analysis of FNP in the cell culture medium supplemented with 20% of FBS revealed that FNP forms a stable and homogenous solution composed of dimer particles associated with one smaller protein with the overall average particle size of about 180nm compared with FNP in water of about 90nm. This was in accordance with the results of Su et al. [38] who found that $C_{60}(OH)_x$ was much more stable in the cell culture medium with serum, in contrast to serum free medium in which aggregation occurs after a few hours. Our results of the DLS study of FNP are also in accordance with the results of Assemi et al. who showed that the aggregation FNPs were significantly increased in solution with higher ionic forces [39].

Cytotoxicity of fullerene depends on its chemical modification, concentration, a cell type and treatment duration [38]. According to literature data, FNP is not cytotoxic to the limit of its solubility [40]. Our results also showed that cytotoxicity of $C_{60}(OH)_{24}$ on PBL at concentration range from 8.86 μM to 443.2μM depends on its concentration, treatment duration, and the cell cycle phase. For evaluation of genotoxic and antigenotoxic effects of FNP but to avoid its cytotoxic effect, the next step in our experiments was determination of LC_{50} values for FNP under conditions in both MN and CA assays. Based on LC_{50} values, we defined three subcytotoxic concentrations of FNP for further experiments. The LC_{50} values for FNP were at a concentration range from 44.3 μM to 443 μM depending on treatment duration (24h or 4h) and on total duration of incubation per assay. This is in correspondence with LC_{50} values for $C_{60}(OH)_{24}$ on human dermal fibroblast HDF through 48h treatment, where LC_{50} was higher than 5g/l (443 μM) according to Sayes et al. [40].

Decreased MN and CA frequency in all cell cycle phases of human lymphocytes confirmed the absence of genotoxicity of fullerenol nanoparticles at subcytotoxic concentrations. The absence of genotoxic effects of FNP at almost the same concentration range used in our study was also found on CHO-K1 cell line by MN and CA assays [29] and on human breast cancer cell lines MCF-7 and MDA-MB-231 by MN and sister chromatid exchange test [27]. Low genotoxicity of FNP was also previously reported by Mori et al. [26] using bacterial reverse mutation assay (Ames test) and chromosomal aberration test in Chinese hamster lung (CHL/IU) cells. They found insignificant increase in the CA frequency at concentrations higher than we used (up to 5000 μg/mL in the presence of S9).

We also examined antigenotoxic effects of FNP using MMC-damaged human lymphocytes as a model. Mitomycin C is known as natural antitumor antibiotic and cytotoxic drug. It induces the DNA interstrand cross-links, which if left unrepaired may block the DNA replication and act highly cytotoxically [41]. At the chromosomal level, MMC produces chromosome aberrations in the S phase-dependent mechanism. Damaging effects of MMC involve ROS generation, which is able to cause oxidation of the DNA, the DNA strand breaks or lipid peroxidation-mediated DNA adducts. In our study, we used 1.5nM of MMC for all endpoints, because higher concentrations induce very high frequency of MN and CA, which is difficult to score accurately (data not shown). Our results showed decrease of MN and CA frequency in

MMC-damaged human lymphocytes. These experimental findings indicate that FNP is a particle with antigenotoxic potential.

We propose that decreasing of MN and CA frequency in PBL, as well as in MMC-damaged human lymphocytes could be explained by capability of FNP to reduce ROS production. Namely, FNP possesses free radical scavenging and antioxidative modulating activity [42]. It is considered that some fullerene derivatives at low concentrations have antioxidative properties while at high concentrations they may express pro-oxidative activity and induce inflammation processes [20]. Fullerenol $C_{60}(OH)_{24}$ scavenges the DPPH, and the reactive oxygen species (ROS) superoxide radical anion ($O_2^{\cdot-}$), singlet oxygen, and hydroxyl radical (HO^{-}), can also efficiently inhibit lipid peroxidation *in vitro* [43, 44]. $C_{60}(OH)_{24}$ acts as a mitochondrial protective antioxidant with direct radical scavenging activity and indirect antioxidative activity [5]. FNP can protect cells against H_2O_2-induced oxidative damage, may stabilize the mitochondrial membrane potential and reduce intracellular ROS production [16]. FNP expresses its protective mechanism through formation of FNP-iron complex therefore disabling further cell damages by ROS [45]. Pre-treatment of irradiated human erythroleukemia K562 cells with fullerenol nanoparticles increases superoxide dismutase and glutathione peroxidase activity and might be due to capability of FNP to modulate antioxidative activity [7, 42].

Also, due to its antioxidative capacity, FNPs have a potent anti-inflammatory activity in a model of acute inflammation in rats using the carrageenan-induced rat footpad edema test [46]. Moreover, polyhydroxylated C_{60} at nanograms' concentrations shows the ability to inhibit the basic inflammatory response [47].

The type of chromosomal aberrations induced by genotoxic agents are cell cycle dependent, and agents are usually categorized as S-phase-dependent or S-phase-independent [48]. Majority of the chemically induced aberrations are formed only during the S phase of the cell cycle and they are mainly chromatid-type aberrations [49]. According to Su et al. [38] $C_{60}(OH)_x$ can reduce metabolic activity in CHO and Chinese hamster lung cells in a G1 phase of the cell cycle after 48 h treatment with 0.1 mg/ml $C_{60}(OH)_x$. They also found decrease of metabolic activity in the S and G2/M phases [38]. Our results showed that FNP slightly increased proliferation capacity of PBL in all phases of the cell cycle. Difference between our results and the results of Su et al. might be caused by different experimental models and the cell origin – human cells *vs.* rodent cells. Namely, in our experimental model, the FNP treatment with nearly the same concentration (0.125 mg/ml in 24h/G1 and 4h/G1/S/G2/M phases of the cell cycle) was shorter than in the Su et al. model. Further, we found that the NDI values were decreased in treatment with FNP on MMC-damaged lymphocytes. It is known that MMC slows down the rate of cell proliferation by covalent binding to the DNA producing monoadducts and the DNA-DNA, and the DNA-protein crosslinks. Adducts interfere with the DNA replication, induce damages leading to reduction in the rate of the DNA replication and to a dose-dependent delay in the cell cycle progression [50]. We suppose that decreased NDI values in our study were the result of MMC activity rather than the FNP impact.

5. Conclusion

Our results showed that fullerenol nanoparticles in the cell culture medium with 20% FBS built homogenous and stable solution with predominant nanoparticles of about 180nm and 90nm respectively. Each nanoaggregate contained two FNPs and the third NP, which was probably a protein from serum.

Regarding cytotoxicity, FNP in higher concentrations induced the cell death. LC_{50} values were in range of micromolar concentrations. In the range of subcytotoxic concentrations FNP did not express genotoxic potential.

The frequency of micronuclei and chromosomal aberrations were reduced under the FNP treatment and regarding a type of chromosomal aberrations, chromatid type of chromosomal aberrations was dominant. On MMC-damaged lymphocytes, FNP revealed antigenotoxic capacity, which was presented by the decreased MN and CA frequency.

Considering the absence of genotoxic and presence of antigenotoxic effects of fullerenol $C_{60}(OH)_{24}$ nanoparticles on human lymphocytes, as well as literature data about its antioxidative

and anti-inflammatory properties at low concentrations, we may conclude that the range of FNP antigenotoxic effect on PBL corresponds with subcytotoxic concentrations defined in our paper.

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