Protective effects of orally applied fullerenol nanoparticles in rats after a single dose of doxorubicin

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Abstract
Polyhydroxylated, water soluble, fullerenol C₆₀(OH)₂₄ nano particles (FNP) in vitro and in vivo models, showed an expresssive biological activity. The goal of this work was to investigate the potential protective effects of orally applied FNP on rats after a single dose of doxorubicin (DOX) (8 mg/kg (i.p.)) 6 h after the last application of FNP. After the last drug administration, the rats were sacrificed, and the blood and tissues were taken for the analysis. Biochemical and pathological results obtained in this study indicate that fullerenol (FNP), in H₂O:dimethyl sulfoxide (DMSO) (80:20, w/w) solution given orally in final doses of 10, 14.4, and 21.2 mg/kg, three days successively, has the protective (hepatoprotective and nephroprotective) effect against doxorubicin-induced cytotoxicity via its antioxidant properties.

Keywords: Fullerenol ● Nano particles ● Doxorubicine ● In vivo ● Oral application

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Doxorubicin (DOX) is an anthracycline antibiotic, one of the most effective and commonly used antineoplastic agents, with a very wide spectrum of antitumor efficacy [1]. One major mechanism underlying the antineoplastic action of DOX concerns its ability to intercalate into the DNA helix and/or bind covalently to proteins involved in DNA replication and transcription (topoisomerase II) [2]. DOX can also interact with mitochondria and bind to cardiolipin blocking the binding of mitochondrial creatine kinase to mitochondrial membranes [3]. Additionally, the increase in DOX redox cycling by complex I of the mitochondrial respiratory chain leads to an increase in reactive oxygen species (ROS) production. These DOX derivates react with oxygen and form superoxide radicals and/or DOX reacts with iron generating H₂O₂ [4]. The mechanism of DOX-induced cardiotoxicity also includes expression of nitric oxid synthases, changes in calcium homeostasis and alteration of molecular signalling [5,6]. All these interactions lead ultimately to cell death.

The clinical usefulness of DOX is restricted, due to several acute and chronic side effects, especially a dose-dependent myocardial injury, which can cause conge stive heart failure. This anticancer drug also affects other organs like the brain, kidneys, liver and skeletal muscles [7,8].

Several attempts have been made to attenuate DOX side effects, including dosage optimization, combined therapy with antioxidants and/or compounds with antiapoptotic activity, development of efficient delivery systems, as well as synthesis and use of analogues [9].

Fullerenes are closed spherical molecules, shaped like a soccer ball and made entirely of carbon atoms. Functionalized fullerenes are one of the many different classes of compounds that are currently being investigated in the rapidly emerging field of nanoscience, especially nanomedicine [10-12]. Chemical modification of fullerenes, C₆₀, into water soluble polyhydroxylated nanoclusters, make them interesting for biological research. The results of latter research in this area suggest that the predominant forms of fullerenol in aqueous environment are clusters with a mean size of 100 nm. The size evolution of fullerenol clusters is dynamic and is sensitive to changes in environmental conditions like pH and temperature [13].

Fullerenols, C₆₀(OH)ₙ, polyhydroxylated derivatives of fullerene C₆₀, are being extensively investigated because of their great potential as antioxidants. Especially, FNP, administered intraperitoneally or intravenous, in vitro and in vivo models, showed an expressive biological activity. FNP might act as antioxidative [14], cell protective [15], radioprotective [16], and geno-protective agent [17], cardio and hepatoprotector in doxorubicin-induced stress in vivo models [18]. In current
literature there are no available data on the effects of orally applied fullerenol.

Yin et al. have found that molecular as much as supramolecular properties of watersoluble derivatives of fullerenol, involving aggregation into nanoparticles/ nanostructures, have a significant impact on their biological activities. In addition, size may influence the distribution of nanoparticles in cells and in tissues [19].

The results of Jing-Ying et al. [20] showed that pretreatment with fullerenol (C₆₀(OH)ₓ, x: 3–24) could protect tissues against CCl⁴-induced oxidative stress in rats by improving the antioxidant ability. Our recent experiments indicate that oral application of FNP (10.0, 14.4 and 21.2 mg/kg) 60 min before i.p. administration of CCl⁴ (0.5 ml/kg) did not have significant influence on serum activity of alanine aminotransferase and aspartate aminotransferase, as well as the concentration of blood urea and creatinine. The histological studies of the heart and liver indicated that they were not damaged by CCl⁴ (unpublished data).

The scope of this experiment was to investigate the potential protective effects of orally applied FNP (dissolved in NaCl (0.9%):DMSO (80:20 w/w) solution) in rats after a single dose of intraperitoneal application of DOX.

MATERIALS AND METHODS

Chemicals and reagents

Fullerenol (C₆₀(OH)₂₄) was synthesized and characterized from polybrominated derivative C₆₀Br₂₄ that was synthesized in a reaction of C₆₀ in Br₂ with FeBr₃ as the catalyst [21]. Fifty (50) mg of C₆₀Br₂₄ was mixed in 5 cm³ 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 cm³ portions of 80% ethanol. Water solution (20 ml) of fullerenol with residual amounts of NaOH and NaBr was applied to the top of the combined ion exchange resin (20 g DOWEX MB50 QC121815 R1) and eluted with demineralized water until discoloration. The water solution was evaporated under low pressure; and a dark brown powder substance left. Fullerenol, involving aggregation into nanoparticles/nanostructures, is highly orientated pyrolytic graphite (HOPG). Figure 1 shows tapping images of large particle of fullerenol in H₂O:DMSO (80:20 w/w) solution. Change of FNP dimensions and particle size distribution in H₂O:DMSO (80:20 w/w) solution did not occur after addition of serum 120 min later at 25 °C or after temperature variation at 37 °C. A multimode quadruplex SPM with NanoScience-Team Nanotec GmbH SNC (Solid Nitride Cone) AFM probe, with the tip radius lower than 10 nm. The used surface was highly oriented pyrolytic graphite (HOPG). Figure 1 shows tapping images of large particle of fullerenol in H₂O:DMSO (80:20 w/w) solution.

Physical determination of Fullerenol (C₆₀(OH)₂₄)

FTIR: C₆₀(OH)₂₄ has following characteristic peaks at 3427, 1627, 1419, 1080 cm⁻¹, 13C-NMR (D₂O); singlet peaks at δ169.47 ppm and multiplet peak at δ160–110 ppm. MALDI MS (m/z): 720 (C₆₀O), 993 (C₆₀(OH)¹⁶), 1043 (C₆₀(OH)²₄), 1061 (C₆₀(OH)²₅), 1128 (C₆₀(OH)²₆), DTG, DTA and TG show two thermal changes, in a temperature range of 120–395 °C, which corresponds to the loss of mass of 35.7% (23.7 OH groups) and at a temperature of 430 °C the evident loss of mass was 64.3% (this is the sublimation temperature of C₆₀). Elementary analysis of FNP provided the following: C – 63.0%, H – 2.0%, which coincides with theoretically calculated values: C – 63.83%, H – 2.13%.

Particle size distribution and zeta potential of FNP in H₂O:DMSO (80:20 w/w) solution are presented in Table 1 (Zetasizer Nano, Malvern Instruments). Ten percent, d(0.1), of particles have dimensions in the range of 33–63 nm; fifty percent, d(0.5), of particles have dimensions in the range of 60–87 nm and ninety percent, d(0.9), of particles have dimensions in the range of 105–132 nm.

Table 1. Particle number distribution, d (nm), and zeta potential, ζ (mV), of FNP in H₂O:DMSO (80:20 w/w) solution

<table>
<thead>
<tr>
<th>FNP Concentration, mg/ml</th>
<th>d(0.1)</th>
<th>d(0.5)</th>
<th>d(0.9)</th>
<th>ζ</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.0 mg/ml</td>
<td>33</td>
<td>60</td>
<td>105</td>
<td>-20.0</td>
</tr>
<tr>
<td>14.4 mg/ml</td>
<td>63</td>
<td>87</td>
<td>132</td>
<td>-14.9</td>
</tr>
<tr>
<td>21.2 mg/ml</td>
<td>63</td>
<td>87</td>
<td>132</td>
<td>-12.8</td>
</tr>
</tbody>
</table>

These parameters corresponds to DLS measuring of fullerenol nanoparticles in H₂O:DMSO (80:20 w/w) solution. Change of FNP dimensions and particle size distribution in H₂O:DMSO (80:20 w/w) solution did not occur after addition of serum 120 min later at 25 °C or after temperature variation at 37 °C. A multimode quadruplex SPM with NanoScience-Team Nanotec GmbH SNC (Solid Nitride Cone) AFM probe, with the tip radius lower than 10 nm. The used surface was highly oriented pyrolytic graphite (HOPG). Figure 1 shows tapping images of large particle of fullerenol in H₂O:DMSO (80:20 w/w) solution.

The large particle about 95 nm consist of a few (5–7) smaller particles with the size of about 25 nm. Particles had nearly spherical shape as can be seen in Figure 1d. The peak-to-valley measurements showed that maximum peak height was at about 4.5 nm and smaller peaks were at the 2.0, 2.5. 3.5 2 nm (Figure 1c).

DOX (Adriablastina®) for i.v. administration was obtained from Pharmacia & Upjohn (Milan, Italy).

The solution for the i.p. application was dissolved in a sterilized and pyrogenic 0.9% NaCl solution (2 mg/ml) inside a laminar flow cabinet immediately before use.

Animals models

Male Wistar rats (Military Medical Academy, Belgrade, Serbia) were obtained at 7 weeks of age, quarantined and housed 3 per cage at a 22–23 °C room temperature, 55±10% humidity and a 12 h light/dark
cycle. They had free access to a standard laboratory diet and water. All experiments were conducted in accordance with the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (ETS 123).

**Statistical analysis**

The data were analyzed using analysis of variance (ANOVA) factorial experiment. Comparison of mean values of measured parameters was performed by Duncan’s multiple range test, for the level of significance $p < 0.05$. The results are shown graphically. For each analyzed parameter is given the least significant difference, LSD. Values for each treatment that are marked in the tables with the same letter are not significantly different for $p < 0.05$ level of significance. The highest value is indicated by the letter “a”, then the values decrease in alphabetical order. The graph shows the standard deviation for each treatment.

**Experimental design**

Untreated control group were treated (o.a.) three days successively with saline only. Tree groups of animals were treated (o.a.) three days successively with FNP in H$_2$O:DMSO (80:20 w/w) solution in dose of: 10, 14.4 and 21.2 mg/kg, respectively. One group of animals were treated (i.p.) with DOX in a single dose of 8 mg/kg. Protected group of rats were treated (o.a.) three days successively with FNP in H$_2$O:DMSO (80:20 w/w) solution in dose of 10 mg/kg, and after 6 h of last dose were treated with (i.p.) DOX 8 mg/kg. All animals were sacrificed after 48 h by urethane anesthesia.

The animals were randomly divided into six groups for six animals per group (Table 2).

I – oral application of 0.9% NaCl solution three days successively (control group);
II – oral application of FNP in H₂O:DMSO (80:20, w/w) solution in final dose of 10 mg/kg, three days successively (operating control group);

III – oral application of FNP in H₂O:DMSO (80:20, w/w) solution in final dose of 14.4 mg/kg, three days successively;

IV – oral application of FNP in H₂O:DMSO (80:20, w/w) solution in final dose of 21.2 mg/kg, three days successively;

V – FNP/DOX group – rats received FNP (o.a.) 10 mg/kg, 30 min before DOX 8 mg/kg (i.p.), three days successively; 6 h after the last FNP application, rats receive DOX 8 mg/kg (i.p.), at once (protected group),

VI – DOX group – rats received DOX 8 mg/kg (i.p.), at once.

Body weight and coefficient of liver weight

The coefficient of heart and liver to body weight was calculated as the ratio of organ tissues (wet weight, mg) to body weight (g). During autopsy, the hearts and livers were excised and weighed. Samples of every heart and liver were fixed in 10% buffered formalin for histological analysis. All tissue samples were embedded in paraffin. Sections (3 µm in thickness) of the embedded tissue were stained with hematoxylin-eosin and periodic acid schiff (PAS) for light microscopy observations, as it was done in previous research [22].

During treatment period, the body weight of each rat was checked. After sacrifice, the body weight was measured and various tissues/organs were collected. Table 3 shows body weight and the coefficients of liver expressed as mg (wet weight of tissues)/g (dead body weight).

Biochemical analysis

Blood for analysis was taken via heart puncture after opening the thoracic region. Serum was used for analysis of enzymatic activity (ALT and AST) and for analysis of urea blood level. All analyses were done in accordance with previously used protocols [23,24]. Each kidney and liver was removed from the sacrificed animals and placed into an ice-cold solution. The adipose tissues were trimmed off. Each organ was minced and homogenized in a Tris-buffer solution (pH 7.4; organ:buffer, 1:10, w/w). MDA determination in liver and kidney tissues were analyzed according to the protocol [23].

AFM Analysis

Multimode quadrex SPM with Nanoscope IIIe controller (Veeco Instruments, Inc.), operated under ambient conditions was used in this work. 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. Used surface is HOPG.

RESULTS

The final body weights of the FNP/DOX and DOX treated animals (V and VI group) were less when compared to the untreated control group of rats. For all groups measured in comparison to the untreated control group (I), the coefficient is not significantly different except for the FNP (oral application in dose of 21.2 mg/kg) and for the treated group (IV) (Figure 2).

Rats received i.p. DOX (VI) in a single dose had significantly higher levels of serum ALT compared with all other groups. Animals from the group protected by a three-day oral application of FNP (V) and from operating control group (orally administrated FNP, group II) had ALT levels comparable to the control group that received saline only (I) (Figure 3).
Levels of AST in serum did not change significantly in all examined groups of animals (Figure 4).

The significant increase of urea serum levels was measured in the group that received i.p. DOX in a single dose (VI), while the values of urea concentration in protected group FNP/DOX (V) and operating control group FNP (II) was comparable to the control group animals treated only with saline (I) (Figure 5).

It was evident that there was a significant increase of MDA level in the liver of rats received i.p. DOX in a single dose (VI). MDA levels in protected group FNP/DOX (V) and operating control group FNP (II) was comparable to the control group animals who received saline only (I) (Figure 6).

The significant increase in kidney MDA level was obtained in the group that received i.p. DOX in a single dose.
dose (VI), and also in protected group FNP/DOX (V), although the MDA level in the protected group was less than in DOX-treated group (Figure 7). MDA level in operating control group FNP (II) was comparable to the control group of animals who received saline orally (I).

**DISCUSSION**

The analyses of ALT serum level in animals that were treated with i.p. DOX in a single dose showed a significant increase compared with values obtained for all other treated groups of animals (Figure 3). Group
protected from acute toxicity of DOX with three days successfully oral application of FNP, and the other groups of animals that also received orally FNP for three days, had ALT levels comparable with the control group. These results indicate a hepatoprotective action of orally inoculated FNP which is in compliance with the published data of i.p. application of FNP [18,22,24,25].

The AST serum levels in animals that received i.p. DOX, only, was correlated with the control group AST level (Figure 4). AST values in groups protected from acute DOX toxicity with o.a. of FNP as well as the group of animals that received only FNP orally for three days had lower AST levels compared with control group. Very high levels of AST in serum of control animals group may be the consequence of stress during the manipulation of animals or the error in experimental analysis. Obtained values indicate that the three day oral pretreatment with FNP had a hepatoprotective effect from acute toxicity caused by single i.p. dose of DOX.

The significantly higher values of urea in rats serum were measured in the group of animals treated with i.p. DOX in a single dose compared with control group (Figure 5). The animals protected from DOX acute toxicity with oral application of FNP for three days and the group of animals received FNP dose orally, also for three days, had the same urea levels as the control group. Obtained values indicate nontoxic and nephroprotective effects of orally administrated FNP (in the final dose of 10 mg/kg) from acute i.p. DOX application (in the final dose of 8 mg/kg). The results are in accordance with previously published data regarding nephroprotective action of i.p. applied FNP [23].

Liver damage caused by i.p. application of DOX in a single dose of 8 mg/kg was manifested two days after treatment as a significant increase of liver MDA levels compared with control animals group (Figure 6). MDA values in liver tissue of animals pretreated for three days with oral application of FNP were significantly reduced compared with previously mentioned DOX group (VI) and were in correlation with control (I). Oral administration of FNP for three days in dose of 10 mg/kg did not cause any toxic effect on liver and had a protective role against DOX applied i.p. in a single dose of 8 mg/kg. These MDA level measurements suggest hepatoprotective action of orally applied FNP from acute DOX toxicity and are compatible with data about hepatoprotective effect of i.p. and i.v. administrated FNP.

Single dose application of DOX increases twice the level of kidney MDA compared with control (Figure 7). Oral pretreatment with FNP decreased MDA level in comparison with a group of animals treated only with DOX. The MDA values in rats that received oral dose of FNP for three days were in correlation with MDA levels of control group. Orally applied FNP (for three days in dose of 10 mg/kg) did not cause any toxic effect on kidney and showed a protective role against i.p. cytotoxicity induced by DOX applied i.p. (in a single dose of 8 mg/kg). Obtained results indicate nephroprotective effect of o.a. FNP and are also consistent with the protective action of i.p. administrated FNP against nephropathy [23,26]. These data show better hepatoprotection than nephroprotection from acute DOX toxicity as a result of antioxidative action of oral pretreatment with FNP for three days.

Oral application of FNP for three days in final doses of 10, 14.4 and 21.2 mg/kg did not cause any visible pathological changes in tissues of kidney, liver and small intestine. There was no evidence of pathological changes in the intestinal mucosa. Single-layer cylindrical epithelium was completely preserved without any inflammatory changes in the lamina propria (mucous membrane) in all examined tissue samples. Pathological changes of renal parenchyma were not observed in any analyzed group. Examined cortical structures had preserved glomerulus as well as proximal and distal tubules. Medullary collecting ducts also had normal lumen with no content. Pathohistological analyses of samples from the sixth (VI) group showed hepatocytes characterized by fine-grained cytoplasm, as an indication of developed parenchymatous degeneration, initial but also reversible pathological process. Liver preparations of groups II–V indicated the absolute preservation of parenchyma, hepatocytes had normal morphology, without present forms of parenchymatous degeneration, which is consistent with the control group. Oral application of FNP in H2O:DMSO (80:20, w/w) solution in final doses of 10, 14.4, and 21.2 mg/kg, three days successively, in all analyzed tissues (liver, kidney and small bowel segment) shows protective effect against citotoxicity caused by DOX administrated i.p. in a single dose of 8 mg/kg.

CONCLUSION

All previously published investigations with FNP as an organo-protector against cell damage induced by a single dose of DOX were established to evaluate protective potential of parenteral administration of FNP. These results are the first evidence of cytoprotective activity of orally applied FNP, in H2O:DMSO (80:20 w/w) solution, as a pretreatment, in final doses of 10, 14.4, and 21.2 mg/kg, three days successively toward acute injury caused by single dose of DOX. Results obtained in this study indicate that fullerol (FNP) in H2O:DMSO (80:20, w/w) solution in final doses of 10, 14.4, and 21.2 mg/kg, three days successively, given orally, may play a hepatoprotective and nephroprotective role in doxorubicin-induced hepato and nephrotoxicity via its antioxidant properties. Further investigations should be directed to determination of orally applied FNP poten-
tial as an organoprotector, as well as the pathways of its activity.

**Abbreviations**

AST – Aspartate aminotransferase  
DLS – Dynamic light scattering  
DMSO – Dimethyl sulfoxide, (CH₃)₂SO  
DOX – Doxorubicin, (ALT)–alanine aminotransferase  
FNP – Fullereneol, C₆₀(OH)₂₄ₙ, nanoparticles  
HOPG – Highly orientated pyrolytic graphite  
i.p. – Intraperitoneal application  
MDA – Malondialdehyde, AFM-atomic force microscopy  
o.a. – Oral application

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IZVOD
ZAŠTITNI EFEKAT ORALNO UNETIH FULERENOLSKIH NANOČESTICA NA PACOVE NAKON JEDNOKRATRATNE PRIMENE DOKSORUBICINA

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(Naučni rad)

Polihidroksilovane, vodorastvorne nanočestice fulerenola C60(OH)24 (FNP) u in vitro i in vivo modelima pokazale su da poseduju višestruke biološke aktivnosti. Dokсорubicin (DOX) jeste antraciklinski antibiotik, i jedan od najefikasnijih anti-neoplastičnih agenata sa veoma širokim spektrom delovanja. DOX ispoljava niz akutnih i hroničnih neželjenih dejstava, naročito na srce, ali i na druge organe: mozak, bubrege, jetru, skeletnu muskulaturu. Cilj ovog eksperimenta bio je da se ispita potencijalni protektivni efekat oralno aplikovanog fulerenola kod pacova nakon jednokratne intraperitonealne (i.p.) primene dok sorubicina (DOX) u dozi od 8 mg/kg i to 6 sati nakon poslednje aplikacije fulerenola. Pacovi koji su primili jednokratnu (i.p.) dozu DOX-a imali su statistički značajno veće vrednosti sermske alanin-aminotransferaze (ALT), kao i veće vrednosti nivoa uree u krvi, u poređenju sa ostalim grupama. Životinje iz štićenih (FNP/DOX) grupe (grupa V koja je pre aplikacije DOX-a bila tretirana oralno primenjenim fulerenolom) kao i operativne kontrole (grupa II, tretirana samo fulerenolom) imale su vrednosti nivoa ALT-e i uree u korelaciji sa kontrolnom grupom (grupa I kojoj je oralno aplikovan samo fiziološki rastvor). U svim ispitivanim grupama životinja nije bilo statistički značajnih razlika u nivou serumske aspartat aminotransferaze (AST). Rezultati su pokazali statistički značajno povećanje nivoa malonildialdehida (MDA) u jetri pacova kojoj je jednokratno aplikovan samo DOX. Nivo MDA u štićenoj grupi (FNP/DOX) i operativnoj kontrolnoj grupi bio je u saglasnosti sa rezultatima dobijenim za kontrolnu grupu (grupa I). Statistički značajan je porast vrednosti MDA u bubrežima u grupi tretiranoj DOX-om u odnosu na štićenu grupu. Vrednosti MDA u kontrolnoj operativnoj grupi u korelaciji su sa onima dobijenim za kontrolnu grupu koja je primila oralno samo fiziološki rastvor. Oralna aplikacija FNP u toku perioda od tri dana u dozama od 10, 14,4 i 21,2 mg/kg nije izazvala nikakve vidljive patohistološke promene na tkivima bubrega, jetre i tankog creva. Biohemijeske i pathohistološke analize su pokazale da FNP primenjen oralno u svim navedenim dozama ispoljava protektivnu aktivnost na hepatoskupljinu i nefrotoksičnost koje su izazvane jednokratnom (i.p.) primenom DOX-a u dozi od 8 mg/kg.