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Fullerenol $C_{60}(OH)_{24}$ prevents doxorubicin-induced acute cardiotoxicity in rats

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Abstract:
Results obtained *in vitro* suggested that fullerenol's antiproliferative properties and protective effects against doxorubicin (DOX) cytotoxicity are mediated by antioxidative and hydroxyl radical scavenger activity. The aim of this study was to examine the influence of fullerenol on acute cardiotoxicity after the administration of a single high dose of DOX *in vivo*. The experiment was performed on male Wistar rats randomly divided into five groups, each containing eight individuals, that were treated as follows: **I**) 0.9% NaCl, **II**) 10 mg/kg DOX, **III**) 50 mg/kg fullerenol 30 min before 10 mg/kg DOX, **IV**) 100 mg/kg fullerenol 30 min before 10 mg/kg DOX, and **V**) 100 mg/kg fullerenol. A functional, biochemical, hematological, and pathomorphological examination of the heart as well as an evaluation of oxidative stress parameters was conducted on days 2 and 14 after DOX administration. The function of the heart was investigated by monitoring heart contractility after the adrenaline infusion. Fullerenol, applied alone, did not alter basal values of investigated animals. Both doses of fullerenol, used as a pretreatment, did not alter the basal parameters of the animals. The 100 mg/kg dose of fullerenol showed better protection. Considering the mechanisms of DOX toxicity, fullerenol likely exerts its protective role as a free radical sponge and/or by removing free iron through the formation of a fullerenol-iron complex. Our results suggest that fullerenol might be a potential cardioprotective agent in DOX-treated individuals.

Key words:
doxorubicin, fullerenol, reactive oxygen species, myocardial injury, cardioprotection

Abbreviations: α-HBDH – α-hydroxybutyrate dehydrogenase, ALT – alanine transaminase, ANOVA – analysis of variance, AST – aspartate transaminase, CAT – catalase, CK – creatine kinase, DOX – doxorubicin, ECG – electrocardiogram, ESR – electron spin resonance, FUL – fullerenol, GR – glutathione reductase, GSH-Px – glutathione peroxidase, LDH – lactate dehydrogenase, LSD – least significant difference, RBC – red blood cells, ROS – reactive oxygen species, SOD – superoxide dismutase, TBARS – thiobarbituric acid reactive substances, WBC – white blood cells

Introduction

The anthracycline antibiotic doxorubicin (DOX) is one of the most important antitumor agents. DOX has been used for more than 30 years for the treatment of various malignancies, including tumors in breast tissue, the bile duct, endometrial tissue, the esophagus

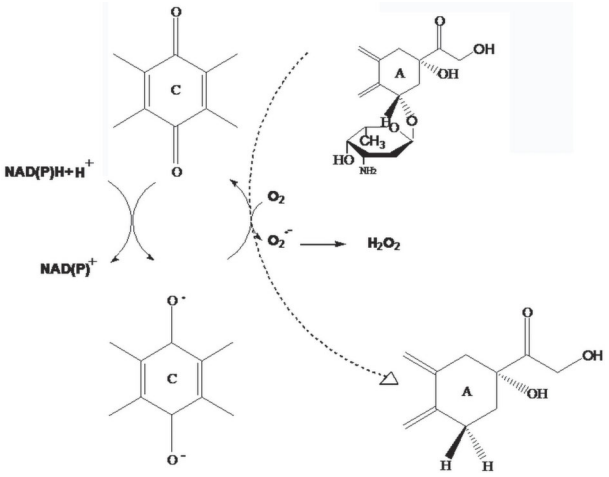

Fig. 1. Redox cycle of doxorubicin

and the liver as well as osteosarcomas, soft tissue sarcomas and non-Hodgkin's lymphoma. However, adverse effects such as myelosuppression and cardiotoxicity limit the use of DOX. The most plausible mechanism of DOX toxicity is an increase in free radical production, which induces lipid peroxidation and oxidative damage in cells [29]. Compared to the liver, the cardiac antioxidant defense system is moderate. There is 150 times less catalase (CAT) and four times less superoxide dismutase (SOD) [23] in the heart. In addition, the unique structure of cardiomyocytes, in which 40% of the cell organelles are mitochondria, may explain why anthracycline antibiotics are selectively toxic to the heart. Cardiac mitochondria possess a NADH dehydrogenase on the outer surface of the inner mitochondrial membrane. Reduction of an anthracycline to the corresponding semiquinone by this enzyme produces an extremely high level of oxidative stress because the anthracycline transfers an electron to molecular oxygen and forms superoxide radicals (Fig. 1). Cell membrane lipids are the most common substrates for oxidative attack. Once initiated, peroxidation continues autocatalytically, and has a progressive course that results in structural and functional changes in the heart tissue. The ultimate damage to mitochondria is oxidative damage of the mitochondrial DNA, which interferes with the regenerative capacity of the organelle. Once this irreversible damage occurs, the cardiomyocytes are destined to undergo apoptosis or necrosis, an event that may not occur until months or years after chemotherapy is completed [5, 6, 12]. Therefore, the most significant pathological changes appear in the heart.

Thus, the use of antioxidants as protective agents could be a potential solution for DOX-induced toxicity. Both *in vitro* and *in vivo* studies have shown that water soluble fullerenol $C_{60}(OH)_{24}$, a polyhydroxylated derivative of fullerene C_{60}, has strong antioxidative potential. The results of several studies suggest that the antiproliferative and protective effects of fullerenol against DOX cytotoxicity are mediated through antioxidative and hydroxyl radical scavenger activity [3, 8, 20, 30, 38, 39]. The purpose of this study was to examine the influence of fullerenol in the prevention of acute cardiotoxicity caused by single high dose administration of DOX. Therefore, we performed functional and structural examination of the heart and monitored hematological parameters as well as the serum activity of enzymes relevant to myocardial integrity. The antioxidative potential of fullerenol $C_{60}(OH)_{24}$ was assessed *in vivo* by measuring lipid peroxidation. We also measured the activity of catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase in order to evaluate the cardiac antioxidative defense system. These results will help clarify the chemistry and mechanisms of action of C_{60} derivatives in biological systems.

Materials and Methods

Chemicals

All of the chemicals used in this study were purchased from the Sigma Aldrich Chemical Co. (St. Louis, MO, USA) unless indicated otherwise.

Animal protocols

All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and approved by the Ethical Committee of Medical Faculty of the University of Novi Sad, Serbia for laboratory animal research. The experiments were performed on 6- to 8-week-old male Wistar rats that weighed 180–220 grams. During the experiment, the animals were kept in standard laboratory conditions

(constant room temperature, constant humidity, a 12/12 dark/light cycle hours, food and water *ad libitum*). DOX (Adriablastina®) for *iv* administration was obtained from Pharmacia & Upjohn (Milan, Italy).

Polyhydroxylated fullerenol $C_{60}(OH)_{24}$ was synthesized in alkaline media by complete substitution of bromine atoms from $C_{60}Br_{24}$ [9]. Fifty milligrams of $C_{60}Br_{24}$ were mixed in 5 cm^3 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 cm^3 portions of 80% ethanol. Twenty ml of solution containing approx. 1 mg/ml of fullerenol and residual amounts of NaOH and NaBr was applied to the top of a combined ion exchange resin (20 g DOWEX MB50 QC121815 R1) and eluted with demineralized water until discoloration occurred. A water solution containing fullerenol, c ≈ 0.05 mg/ml, pH = 7, was evaporated under low pressure, and a dark brown powder was left in the beaker for further use.

All solutions for *ip* administration were prepared *ex tempore* by dissolving the substances in sterilized and apyrogenic 0.9% NaCl solution in a laminar flow hood.

The animals were randomly divided into five experimental groups containing eight individuals. The animals received the following treatments: **I**) 0.9% NaCl (control), **II)** 10 mg/kg DOX, **III**) 50 mg/kg fullerenol 30 min before 10 mg/kg DOX, **IV**) 100 mg/kg fullerenol 30 min before 10 mg/kg DOX, and **V**) 100 mg/kg fullerenol. Before sacrificing the animals, they were anesthetized with 25% urethane (4 ml/kg) (Sigma, St. Louis, USA), immobilized in a dorsal position and allowed to breathe spontaneously. The experiments were performed with two replicates and the animals were sacrificed 2 and 14 days after receiving treatment.

Examination of heart contractility

A functional examination of the heart was conducted using an adrenaline test [21]. The cardiac cycle of anesthetized rats was monitored before and during adrenaline infusion. The adrenaline solution (Adrenalin HCl, 1:1000, 1 mg/ml, Jugoremedia, Zrenjanin, Serbia) was infused *via* the jugular vein using an infusion pump (speed rate was 0.172 μg/s) and an electrocardiogram (ECG) was continuously recorded using a six-channel electrocardiograph until the first appearance of reflex bradycardia. The elapsed time from the beginning of the adrenaline infusion to the appearance of bradycardia was used to assess cardiovascular contractility of the heart.

Blood biomarker assay

Blood for hematological and biochemical evaluation was taken by heart punctuation after opening the thoracic region. The blood cell count (erythrocytes and leucocytes), as well as hemoglobin and hematocrit, were determined using a hemocounter (Hematology Analyser 901062, "Arcus Biotron", Wien, Austria).

BIOCHEMICAL ASSAYS

Instruments

To measure enzyme activity and lipid peroxidation, we used the Agilent 8453 UV/VIS spectrophotometer with a thermostated multicell position sample system and biochemical analysis software for assaying enzyme kinetics.

Preparation of heart tissue homogenates

After quickly excising and washing the hearts in a 0.9% NaCl solution and removing extraneous fat connective tissue, the hearts were submerged in liquid nitrogen for 60 s. After removing the tissue from the liquid nitrogen, the hearts were put into TRIS/KCl buffer, pH 7.4, and put in an ultrasonic bath for 2 min in order to obtain a tissue homogenate (10% w/v). Finally, the mixture was homogenized on a Potter-Elvehjem homogenizer. Prior to centrifugation, aliquots for determining thiobarbituric acid reactive substances (TBARS) were taken. The remaining homogenates were centrifuged at 15,000 × g and 4°C, for 15 min. The kinetic measurement of enzymatic activity (CAT, SOD, glutathione peroxidase (GSH-Px) and glutathione reductase (GR)) in the tissue was measured immediately after centrifugation of the supernatants.

Serum enzyme activity

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK), lactate dehydrogenase (LDH) and α-hydroxybutyrate dehydrogenase (α-HBDH) were assayed to evaluate cardiac injury. Serum for the determination of enzyme activity

was prepared by centrifugation (3000 rpm) of previously incubated (37°C, 30 min) blood samples. The enzyme activities (expressed as U/l) for AST, ALT, CK, LDH and α-HBDH were determined *ex tempore* using test reagents from the Randox firm (United Kingdom).

TBARS assay

To measure lipid peroxidation by determining TBARS in heart tissue, the modified method by Uchiama and Mihara [40] was used. An aliquot of 0.5 ml of 10% tissue homogenate (TRIS/KCl buffer, pH 7.4) was added to a mixture of 3 ml of 1% H_3PO_4 (JT Baker, USA), and 1 ml 0.6% thiobarbituric acid (TBA) in an aqueous solution. The mixture was stirred and heated in a boiling bath for 45 min. After cooling, the extraction was carried out with 4 ml of 2-butanol (POCH, Poland) and the organic layer was separated with centrifugation. Optical density of the organic layer was determined with a UV/VIS spectrophotometer set at 535 nm. The TBARS level was expressed as nmol/mg of protein.

Antioxidative enzyme assays

SOD activity was measured by inhibition of superoxide radical production in a xanthine-xanthine oxidase reaction according to the method described by McCord and Fridovich [28]. One unit of activity was defined as the amount of enzyme necessary to decrease the rate of cytochrome c reduction to 50% at 25°C maximum and pH 7.8.

CAT activity was determined by the rate of hydrogen peroxide disappearance measured at 240 nm [4]. One unit of CAT activity was defined as the amount of enzyme that decomposes one mmol H_2O_2/min at 25°C and pH 7.0.

The activity of non-selenium GSH-Px was determined by the glutathione-dependent reduction of *t*-butyl hydroperoxide. As such, we utilized a modification of the assay described by Paglia and Valentine [33]. Specifically, the oxidized glutathione formed by the enzymatic action of GSH-Px is instantly and continuously reduced by an excess of GR added to the assay mixture, thereby providing a constant level of reduced glutathione. The concomitant oxidation of NADPH to NADP was monitored spectroscopically at 340 nm. One unit of GSH-Px activity was defined as the amount needed to oxidize one nmol NADPH/min at 25°C and pH 7.0.

GR activity was determined using the method of Glatzle et al. [16]. This assay is based on NADPH oxidation concomitant with glutathione reduction. One unit of GR activity was defined as the oxidation of 1 nmol NADPH/min at 25°C and pH 7.6.

Protein determination

Protein content was determined with the biuret method using test reagents from the Sentinel Diagnostics firm (Milan, Italy).

Pathomorphological study

In order to evaluate the cardioprotective effects of fullerenol on heart tissue, the animals were sacrificed

Tab. 1. Tissue damage score for myocardial changes (MDS – Myocardial Damage Score)

Degree	Description
0	Normal histological structure
1	Mild changes – sporadic myocytes with cytoplasmatic vacuoles and normal nucleus. Mild dilatation of blood vessels. Perivascular appearance of polymorphonuclear cell infiltrates (PMNCI).
2	Moderate damage – more than 50% of cells showed mild vacuolization of the cytoplasm. The nucleus had a normal structure. Edema, hyperemia and small hemorrhage in the epimysium. Perivascular gathering of polymorphonuclear cell infiltrates (PMNCI).
3	Strong focal damage – more than 50% of cells showed vacuolization of cytoplasm and pyknotic nucleus. Diffuse perivascular gathering of polymorphonuclear cell infiltrates (PMNCI).
4	Strong diffuse damage – pronounced plasmolysis and karyolysis of myocytes. Diffuse perivascular and tissue accumulation of polymorphonuclear cell infiltrates (PMNCI).
5	Activation and proliferation of fibroblasts joined with the production of connective tissue.

on the 2nd and 14th day after the treatment. The apex of the myocardium was removed and tissue slices were fixed in 10% neutral-buffered formaldehyde, embedded in paraffin and sectioned. The sections were stained with hematoxylin and eosin, then analyzed using a light microscope. The level of damage in the tissue was assessed according to a five-point semiquantitative scale, i.e., a tissue score for degenerative and vascular changes (Tab. 1).

Statistical analysis

The statistical significance of the differences noted in the biochemical parameters was evaluated using the one-way analysis of variance (ANOVA) method followed by a least significant difference test (LSD test) as a *post-hoc* analysis. For the histopathological changes that were observed, statistical differences were evaluated by the Kruskal Wallis ANOVA. The p values (< 0.001, < 0.01, < 0.05) are provided in the figure and table descriptions.

Results

Effect of fullerenol on heart contractility

Two days after DOX treatment, no changes in functional status of the rats' hearts were observed in any group. However, 14 days after DOX administration, the latency to the appearance of reflex bradycardia in the ECG record of the DOX-treated group of animals was significantly longer than the latencies for the control group and the groups pretreated with fullerenol. In addition, normal heart contractility was observed in the group treated only with fullerenol (Fig. 2).

Effect of fullerenol on blood biomarkers

The administration of DOX did not significantly change the number of red blood cells (RBC) of the values of hemoglobin and hematocrit two days after the treatment in any treated group. However, 14 days after applying the DOX, the number of red blood cells was significantly lower in animals treated only with DOX compared to the control group. In the experimental groups that were pretreated with fullerenol, as well as the group that was treated only with fullerenol, the number of RBC was at the same level as untreated animals (Fig. 3a). These findings were followed by an evaluation of hemoglobin and hematocrit (Fig. 3b and 3c). The most significant changes were found in the leukocyte count. Two days after the DOX treatment, a statistically significant decrease in the number of leukocytes was found in the group that received DOX and in the group that was pretreated with 100 mg/kg fullerenol. However, two weeks after application, the total number of leukocytes had significantly increased only in the group of rats that were treated with DOX alone. The groups that received fullerenol as a pretreatment, as well as the group treated only with fullerenol, had approximately the same number of white blood cells (WBC) as the control animals (Fig. 3d).

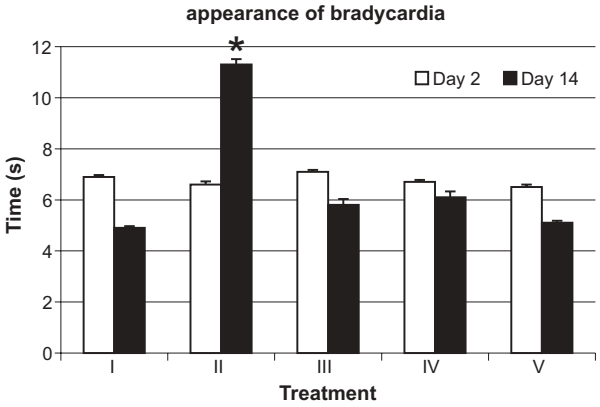

Fig. 2. The influence of fullerenol on the appearance of reflex bradycardia after adrenaline infusion in rats treated with doxorubicin (* $p < 0.05$ related to I; I – control; II – 10 mg/kg doxorubicin; III – 10 mg/kg doxorubicin + 50 mg/kg fullerenol; IV – 10 mg/kg doxorubicin + 100 mg/kg fullerenol; V – 100 mg/kg fullerenol)

Effect of fullerenol on serum enzyme activity

A single high dose injection of DOX induced severe biochemical changes. The preliminary assessment of cellular integrity in the DOX group was done by determining the activity of AST, ALT and LDH. These parameters showed a significant increase in the DOX group two days after treatment relative to the control group. Myocardial damage, two days after application, was observed in the group that received DOX, accompanied by a statistically significant increase in CK and α-HBDH activity. When animals were given 50 mg/kg fullerenol as a pretreatment, the fullerenol did not succeed in protecting heart tissue against cel-

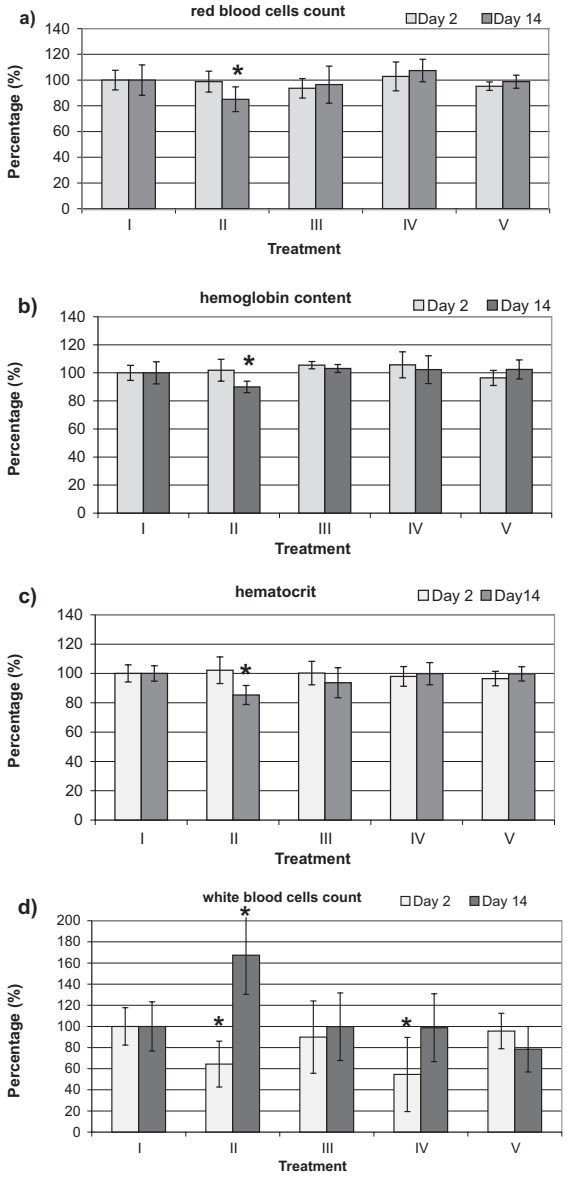

Fig. 3. Influence of fullerenol on blood biomarkers of rats treated with doxorubicin: (**a**) red blood cells, (**b**) hemoglobin, (**c**) hematocrit and (**d**) white blood cells. Results are expressed as a percentage related to control group (* $p < 0.05$ related to control; I – control; II – 10 mg/kg doxorubicin; III – 10 mg/kg doxorubicin + 50 mg/kg fullerenol; IV – 10 mg/kg doxorubicin + 100 mg/kg fullerenol; 100 mg/kg V – fullerenol)

lular damage. As a result, CK, α-HBDH and ALT activity significantly increased two days after DOX treatment. However, the group of animals (IV) protected with a 100 mg/kg dose of fullerenol had enzyme activity similar to the control group. Two weeks after DOX and fullerenol application, the enzyme activity in all treated groups was comparable to the control values. No changes in enzymatic activity were detected in the group that received only a 100 mg/kg dose of fullerenol either 48 h or 14 days after the application regarded to control (Fig. 4a–e).

Effect of fullerenol on lipid peroxidation in heart tissue

Two days after a single dose of DOX, we observed a significantly increased concentration of TBARS in the heart tissue. However, the groups that were pretreated with fullerenol, had a similar level of lipid peroxidation as the control animals. Fourteen days after DOX treatment, the TBARS level, in the group that received only DOX was still significantly higher compared to the control group. The intensity of lipid peroxidation in the myocardial tissue 14 days after DOX treatment was the same as the control group for the groups pretreated with fullerenol. The *ip* application of 100 mg/kg fullerenol did not affect the lipid peroxidation intensity two days or 14 days after treatment (Fig. 5).

Effect of fullerenol on the activity of antioxidative enzymes

Tissue levels of SOD, CAT, GSH-Px and GR were measured (Fig. 6a–d). Application of DOX significantly elevated the activity of all the examined enzymes compared to the control group. High levels of antioxidant enzymes were observed two and 14 days after DOX treatment. Pretreatment with fullerenol 30 min before the application of DOX succeeded in preventing oxidative stress in heart tissue and maintained the basal activity of SOD, CAT, GSH-Px and GR. The dosage of fullerenol in the present study was not toxic to the heart tissue, and the activity of antioxidative enzymes in the fullerenol control group (100 mg/kg, group V) did not reveal any significant changes compared to the control group.

Effect of fullerenol on histopathological examinations of the heart

The damage score analysis of the heart tissue showed both degenerative and vascular changes on the 14th day after DOX treatment (Tab. 2). Histopathological sections of the heart tissue from DOX treated rats, compared to control sections (Fig. 7a), had numerous intracytoplasmic vacuoles that had different shapes and sizes, pyknotic nuclei, edema, hyperemia and small hemorrhage in the epimysium. Furthermore, the

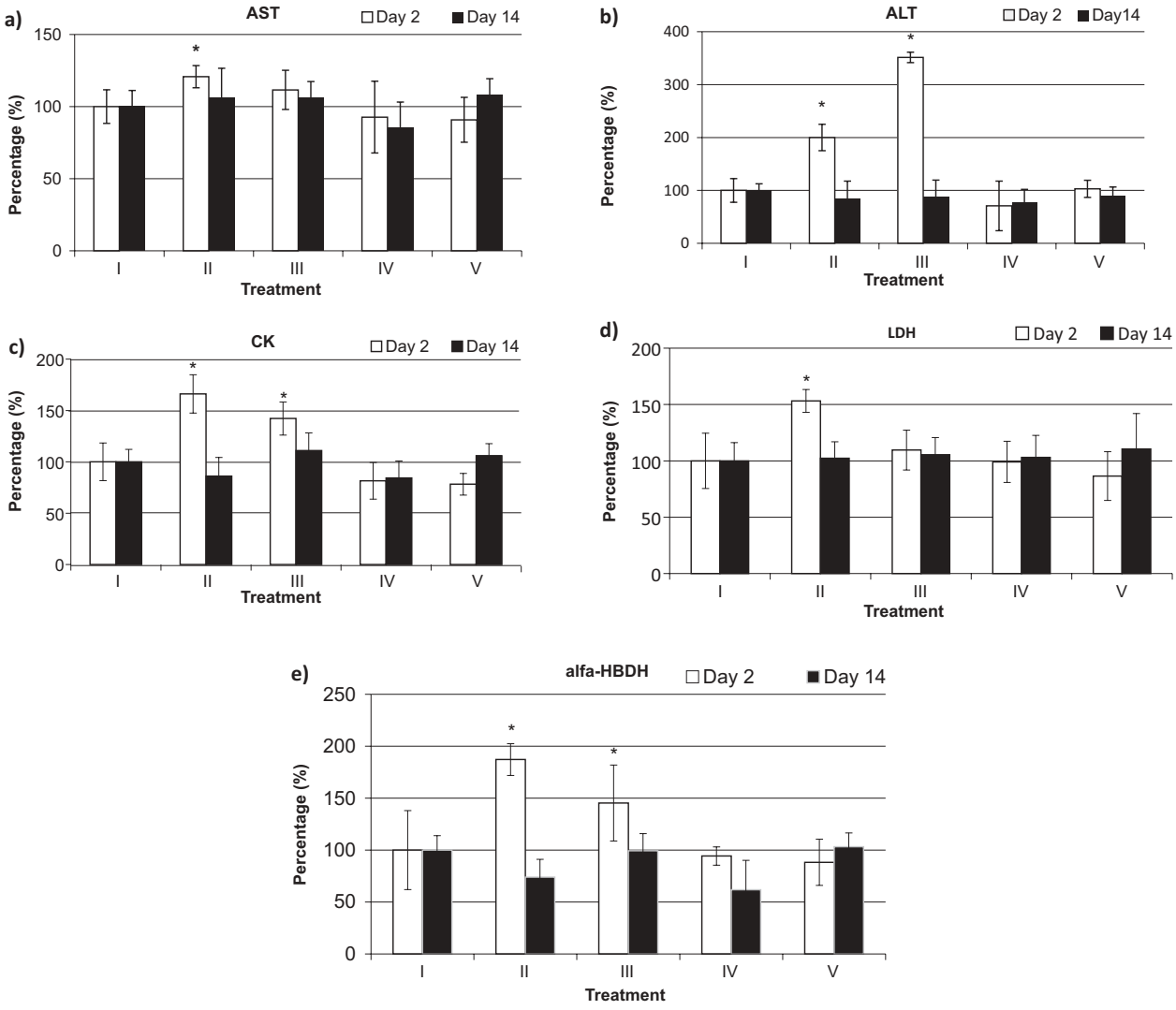

Fig. 4. Influence of fullerenol on enzyme activity in serum of rats treated with doxorubicin: (**a**) AST, (**b**) ALT, (**c**) CK, (**d**) LDH and (**e**) α-HBDH. Results are expressed as a percentage related to control group (*p < 0.05 related to I; I – control; II – 10 mg/kg; doxorubicin III – 10 mg/kg doxorubicin + 50 mg/kg fullerenol; IV – 10 mg/kg doxorubicin + 100 mg/kg fullerenol; V – 100 mg/kg fullerenol)

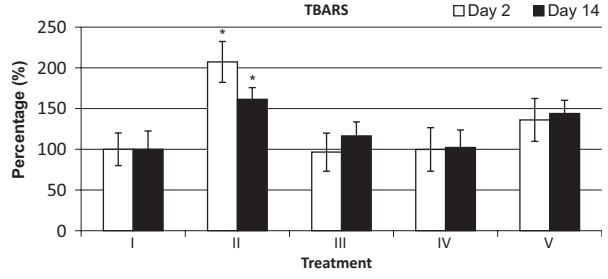

Fig. 5. Influence of fullerenol on TBARS levels of rats treated with doxorubicin. The results are expressed as a percentage related to the control group (*p < 0.05 related to control; I – control; II – 10 mg/kg doxorubicin; III – 10 mg/kg doxorubicin + 50 mg/kg fullerenol; IV – 10 mg/kg doxorubicin + 100 mg/kg fullerenol; V – 100 mg/kg fullerenol)

DOX-treated tissue had dilated blood vessels with discontinued basal membranes surrounded by an accumulation of polymorphonuclear cell infiltrates and the proliferation of fibroblasts along with the production of connective tissue. These changes were most intense in the groups treated with DOX and in the group treated with 50 mg/kg fullerenol and DOX (Fig. 7b, c). However, the application of 100 mg/kg fullerenol prior to DOX administration significantly ameliorates the degenerative changes caused by DOX. In the 100 mg/kg fullerenol group the appearance of small, individual vacuoles was observed in a limited number of endocardial myocytes, while the

Tab. 2. The effects of different treatments on the degree of myocardial damage seven days after the treatment

Treatment		Myocardial damage (8 hearts 4 slices)						$X_{av} \pm SD$
		0	1	2	3	4	5	
Control	25	7	0	0	0	0		0.22 ± 0.42
Dox* 10		0	5	20	7	0	0	$2.40 \pm 0.50\ a^3$
Dox 10 + Full** 50		0	4	22	6	0	0	$2.53 \pm 0.51\ a^3\ c^3\ d^3$
Dox 10 + Full 100		0	22	10	6	0	0	$1.59 \pm 0.50\ a^3\ b^3\ c^2$
Full 100	0	19	13	0	0	0		$1.06 \pm 0.67\ a^2\ b^3$

a^2, a^3 – $p < 0.01$; 0.001 related to control; b^3 – $p < 0.001$ related to 10 mg/kg Dox; c^2, c^3 – $p < 0,01$; 0.001 related to 100 mg/kg Full; d^3 – $p < 0.001$ related to 10 mg/kg Dox + 100 mg/kg Full; * DOX – doxorubicin; ** Full – fullerenol

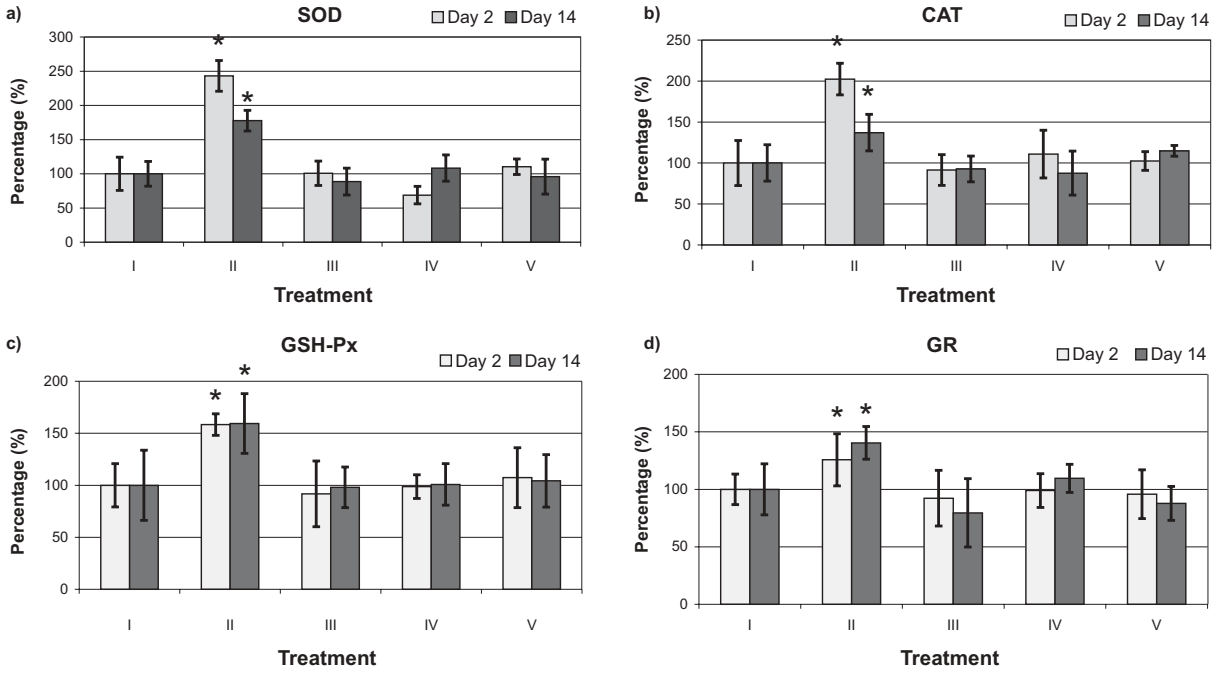

Fig. 6. Influence of fullerenol on the activity of antioxidative enzymes in the heart tissue of rats treated with doxorubicin: (**a**) superoxide dismutase (SOD), (**b**) catalase (CAT), (**c**) glutathione peroxidase (GSH-Px), and (**d**) glutathione reductase (GR). The results are expressed as a percentage related to the control group (*$p < 0.05$ related to control; I – control; II – 10 mg/kg doxorubicin; III – 10 mg/kg doxorubicin + 50 mg/kg fullerenol; IV – 10 mg/kg doxorubicin + 100 mg/kg fullerenol; V – 100 mg/kg fullerenol)

normal structure of the cardiomyocytes was largely sustained with the presence of minor interstitial edema and hyperemia. Focal leakage was also localized only in the inner side of myocardium and in endocardium. Additionally, all blood vessels were just slightly dilated without damage in the basal membranes and were surrounded by only a small quantity of accumulated polymorphonuclear cell infiltrate (Fig. 7d).

Semiquantitative pathohistological analysis confirmed that the higher dosage of fullerenol, given as a pretreatment to DOX, significantly diminished structural changes in cardiomyocytes in response to

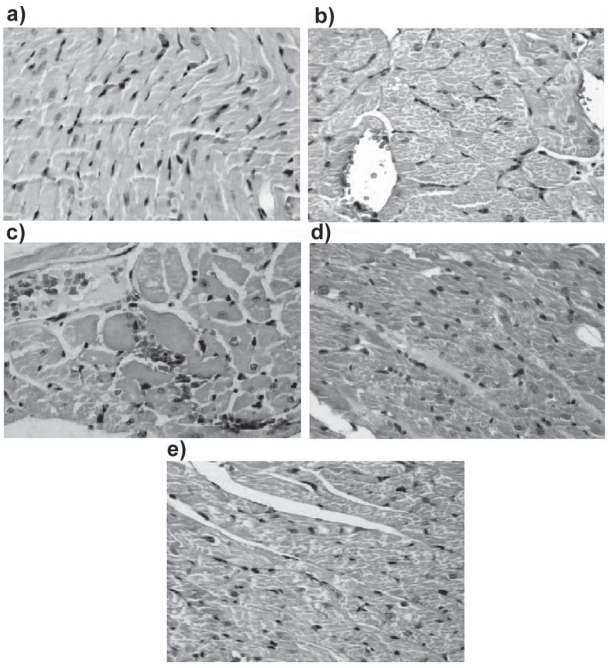

Fig. 7. Histological section of the heart (HE, 40×): (**a**) control group – no histological lesions found; (**b**) group treated with doxorubicin – appearance of numerous vacuoles, total degeneration of normal tissue structure (**c**) group treated with 50 mg/kg of fullerenol 30 min. prior doxorubicin – focal hemorrhage, total degeneration of myocytes; (**d**) group treated with 100 mg/kg of fullerenol 30 min prior doxorubicin – discreet appearance of vacuoles; (**e**) group treated with 100 mg/kg – small vacuoles in central part of sarcoplasm

the toxic effects of DOX. Fullerenol, applied alone, caused the same type of structural alterations that were observed when it was given in combination with DOX, but only in small isolated areas of cardiomyocytes (Fig. 7e).

Discussion

DOX is a very important agent for the treatment of cancer patients. Effective anticancer therapy with DOX and other quinine anthracyclines is severely limited by acute and chronic side effects such as myelosuppression and cardiotoxicity [22, 37]. The acute cardiovascular side effects are characterized by hypotension, tachycardia and various arrhythmias. Sympathomimetic drugs, like adrenaline, may increase peripheral arterial resistance, leading to a marked rise in blood pressure. In the presence of normal cardiovascular reflexes, the rise in blood pressure elicits a baroreceptor-mediated increase in vagal tone with a concomitant slowing of the heart rate [24]. Previous studies have shown that application of DOX causes damage to the heart and baroreceptors, which is indicated by reflex alterations [36]. Our results showed that the reflexes in DOX treated animals were maintained, but their appearance was delayed, which implies the presence of heart damage. Both doses of fullerenol almost completely abolished this DOX-induced disturbance and the latency of adrenaline-induced reflex bradycardia in the ECG record was similar to the control group. This finding also suggested that fullerenol could protect heart function. Administration of DOX caused a severe depletion in the number of blood cells, particularly WBC. Dragojevic previously found that 6 mg/kg and 10 mg/kg doses of DOX caused a strong depletion of WBC within 7 days, and after 14 days, the number of WBC significantly increased compared to control animals [14]. Our results agree with these data. The DOX treated group also had diarrhea and they developed secondary dehydration, which concurred with the high hematocrit values and agrees with prior literature [17]. Both doses of fullerenol exerted a hematoprotective effect on healthy animals treated with DOX and sustained the number of all blood cell types at basal levels. When fullerenol was administrated alone, it did not influence any of the examined hematological parameters.

The serum activity of the CK, LDH, α-HBDH, AST and ALT enzymes are often used as markers of cardiovascular damage. According to previously published papers, multiple increases in the levels of CK, LDH, and α-HBDH in serum were observed as a result of oxidative stress originating from ischemia/reperfusion injury [18, 26], myocardial infarction [2], irradiation [14, 15] and toxicity induced by xenobiotics [7, 11, 25, 41]. In our experiments, significantly increased levels of these enzymes were detected after two days in the groups treated only with DOX, indicating acute myocardial membrane damage. The animals protected by 100 mg/kg fullerenol showed physiological levels of enzymatic activity (CK, LDH, α-HBDH, AST and ALT). Forty-eight hours after the treatment (DOX+FUL), the 50 mg/kg dose of fullerenol maintained normal enzyme activity only for AST and LDH. Fourteen days after DOX treatment, for all experimental groups, the activity of the examined serum enzymes was similar to the control animals. These results were expected because ele-

vated levels of these enzymes usually return to normal within 3–4 days if no other injury has occurred. Therefore, we can conclude that the 100 mg/kg dose has a better protective effect and does not modulate any enzyme activity when it is applied alone even two weeks after treatment.

Earlier publications examined the biological activity of fullerenol to show its high antioxidative potential through the measurement of lipid peroxidation intensity. In the Fe^{2+}/ascorbate system, fullerenol reduced the lipid peroxidation of liposomes when it was applied with different plant extracts [34, 35]. In the model of goat epididymal spermatozoa lipid peroxidation, the peroxidation intensity was significantly lower in samples incubated with fullerenol [31]. Lai et al. also found that administration of fullerenol during an ischemia-reperfusion injury in the small intestine of mongrel dogs diminished intestinal malonyldialdehyde contents [26]. *In vivo* studies of NO-scavenging activity with a model of testicular antioxidant enzymes in rats showed that pre-treatment with fullerenol, prevented the NO-induced decrease in antioxidative enzyme activity after SNP treatment [30]. In our experiments, the intensity of lipid peroxidation was significantly higher in groups treated only with DOX two and 14 days after DOX treatment. Both doses of fullerenol abolished the toxic effect of DOX and maintained lipid peroxidation at a basal level. Applied alone, the 100 mg/kg dose of fullerenol did not cause any alteration in the TBARS level. The activity of SOD, CAT, GSH-Px and GR in our experiments was significantly higher only in the group treated with DOX alone. These results are in accordance with the findings of Yin et al., which reported an elevation of SOD, CAT and GSH-Px activity in the rat heart after the application of DOX [41]. Although DOX can directly suppress the gene expression for these enzymes, DOX can also indirectly upregulate production of antioxidative enzymes by producing reactive oxygen species (ROS) [7, 27, 31, 41]. In our experiments, fullerenol exerted a protective effect in all experimental groups by maintaining the basal activity levels of antioxidative enzymes. These results support the hypothesis that fullerenol is a strong antioxidant and has no observed toxic effects in treated species. Studies of the antioxidative mechanisms of fullerenol showed that fullerenol $C_{60}(OH)_{24}$ has the ability to act both as an iron chelator and as a direct free radical scavenger. Djordjevic et al. proposed a possible mechanism for free radical scavenging in chemical model system using an electron spin resonance (ESR) method [8]. According to this study, fullerenol exhibited a high efficacy for inhibiting the production of hydroxy and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals through the generation of the fullerenol radical $C_{60}(OH)_{23}O$. The interaction between hydroxyl radicals and fullerenol is also based on the addition reaction of 2n $^{\bullet}OH$ radicals to the remaining double bonds of the fullerenol core, yielding $C_{60}(OH)_{24}$ + 2n $^{\bullet}OH$ (n = 1–12). Another study with hydroxyfullerene and metal salts demonstrated that fullerenols react rapidly and irreversibly with a variety of metal salts by forming insoluble metal–hydroxyfullerene cross-linked polymers [1, 19]. The mixed cross-linked complex $(Fe(fullerenol)_n(H_2O)_m)^{3+}[Fe(H_2O)_6]^{3+}$ obtained from the experiment conducted with fullerenol $C_{60}(OH)_{24}$ and Fe^{3+} ion [19] is in accordance with the results of this study. In addition, based on results of the experiment conducted on rats treated with a single high dose of DOX, Djordjevic Milic et al. proposed two mechanisms of fullerenol protection against DOX-induced toxicity in erythrocytes: binding to the hemoglobin sites to keep the site in functional condition and/or making complexes with iron that diminish free radical reactions [10].

The results of our pathohistological analysis of heart tissue conducted in rats after a single dose of DOX parallel similar studies that were previously reported [13, 14, 32]. Cardiac histopathological changes induced by DOX were noticeable with light microscopy on the 14th day after DOX treatment. In all treated groups, statistically significant structural alterations were observed compared to control animals. The most intensive alterations were found in the heart tissue of the rats that were only treated with DOX and in the group of animals pretreated with 50 mg/kg of fullerenol. The damage intensity of the heart tissue in those animals was significantly higher compared to animals treated with 100 mg/kg fullerenol and in the animals treated only with fullerenol. In general, when 100 mg/kg fullerenol was applied as a pretreatment, it provided protection and sustained the structural integrity of cardiac cells in DOX-treated animals. When 100 mg/kg fullerenol was applied alone, it caused mild vascular changes, which are likely reversible. Both applied doses of fullerenol (50 and 100 mg/kg) maintained the majority of the investigated parameters at control levels. This maintenance confirms the protective effect of polyhydroxylated fullerene. Our results support our hypothesis and complete a previ-

ously conducted investigation indicating that fullerenol possesses high antioxidative and cytoprotective potential without any recorded side effects. Considering that the putative mechanisms of DOX toxicity are predominantly based on free radical production as well as the results of the previous investigation and the results obtained in our experiment, we can conclude that the protective role of fullerenol depends on its high antioxidative potential. Regarding the unique electrochemical features of fullerenol, it likely exerts its antioxidant effect by acting as a free radical sponge and/or by removing free iron through the formation of the fullerenol-iron complex and therefore disables further cell damage by ROS. These findings, supported by results obtained in our functional and biochemical examinations, imply that fullerenol might be a potential cardioprotective agent for DOX-treated individuals.

Conflict of interest: None declared.

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