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Short communication

Acute doxorubicin pulmotoxicity in rats with malignant neoplasm is effectively treated with fullerenol $C_{60}(OH)_{24}$ through inhibition of oxidative stress

Rade Injac[1], Natasa Radic[1], Biljana Govedarica[2], Martina Perse[3], Anton Cerar[3], Aleksandar Djordjevic[4], Borut Strukelj[1]

[1]Faculty of Pharmacy, Institute of Pharmaceutical Biology, University of Ljubljana, Askerceva 7, 1000 Ljubljana, Slovenia

[2]Faculty of Pharmacy, Institute of Pharmaceutical Technology, University of Ljubljana, Askerceva 7, 1000 Ljubljana, Slovenia

[3]Institute of Pathology, Medical Experimental Centre, Medical Faculty, University of Ljubljana, Korytkova 2, 1000 Ljubljana Slovenia

[4]Faculty of Sciences, Department of Chemistry, University of Novi Sad, Trg Dositeja Obradovica 3, 21000 Novi Sad, Serbia

Correspondence: Rade Injac, e-mail: injacrade@gmail.com

Abstract:
The aim of this study was to investigate the possible protective role of fullerenol (FLR, $C_{60}(OH)_{24}$ on doxorubicin (DOX)-induced lung toxicity using biochemical and histopathological approaches. Rats (Sprague-Dawley outbred) were randomly divided into five groups. The healthy control group received no medication (saline only). The other four groups had chemically induced breast cancer (1-methyl-1-nitrosourea; 50 mg/kg, *ip*). The second group was the cancer control group (saline only). The other three groups were DOX (8 mg/kg, *ip*), FLR/DOX (100 mg/kg, *ip*, 30 min before DOX; 8 mg/kg, *ip*), and FLR (100 mg/kg, *ip*), respectively.
The levels of malondialdehyde (MDA) and oxidized glutathione (GSSG) in the lung tissue were higher in the group treated with DOX alone than in the control groups. The activities of catalase (CAT), glutathione reductase (GR), superoxide dismutase (SOD), and lactate dehydrogenase (LDH) were found to be increased in the lung tissue of the animals in the DOX group over all the other groups, while GSH-Px significantly decreased in activity compared with the control and FLR groups. There was no significant difference in MDA and GSSG levels and enzyme activities in either control (healthy; cancer) or FLR (FLR/DOX; FLR) groups.
The acute change found in the DOX group was subpleural edema. In contrast, the groups treated with FLR appeared to be virtually histopathologically normal.
In conclusion, this study clearly indicates that DOX treatment markedly impairs pulmonary function and that pre-treatment with FLR might prevent this toxicity in rats through inhibition of oxidative stress.

Key words:
doxorubicin, pulmotoxicity, fullerenol, oxidative stress, lung, rats

Abbreviations: CAT – catalase, DOX – doxorubicin, FLR – fullerenol, GR – glutathione reductase, GSH – glutathione, GSH-Px – glutathione peroxidase, GSSG – oxidized glutathione, LDH – lactate dehydrogenase, MDA – malondialdehyde, MNU – 1-methyl-1-nitrosourea, ROS – reactive oxygen species, SOD – superoxide dismutase, TAS – total antioxidant status, TP – total protein concentration

Introduction

Systematic administration of chemotherapeutic drugs that are toxic to dividing cells is a very important means for the treatment of tumors [7]. Unfortunately, most cytotoxic agents cannot effectively discriminate healthy cells from malignant ones; they rapidly and non-selectively accumulate in healthy tissues, resulting in severe clinical toxicities [12]. One of the most widely employed anticancer agents whose clinical use is limited by acute and chronic cardiotoxicity is the anthracycline doxorubicin (DOX) [8]. Other tissues like kidneys, lungs, liver and skeletal muscles are also affected by DOX [8, 16, 18, 19, 25, 28].

DOX is a cycle-specific agent, which attacks only the cells that are actually in the cell cycle. It acts by inhibiting the enzyme topoisomerase II, which leads to double strand breakages in the DNA double helix and also by intercalating with the DNA [35, 37]. The most frequently investigated effect of DOX on a molecular level is its tendency to generate large amounts of reactive oxygen species (ROS) through the formation of semiquinone type free radicals, which are produced *via* NADPH-depended reductases. Derivates originating from DOX give rise to superoxide radicals by reacting with oxygen [29]. Furthermore, DOX inhibits the ROS neutralizing enzymes, which are normally present in the tissue [20]. The disturbance in oxidant-antioxidant systems results in tissue injury that manifests with lipid peroxidation and protein oxidation in the tissue, which are also the main causes of lung injury [13, 25].

Studies of lung injuries produced by airborne agents and environmental toxins have demonstrated the inflammatory process alongside the elevated cellular concentration of free radicals [3, 14, 26, 33]. ROS have been implicated in many lung diseases including those associated with exposure to asbestos, nitrogen dioxide, ozone, paraquat, hyperoxia, carbon tetrachloride, and the anticancer drugs bleomycin and DOX [32]. Phagocytic cells have also been implicated in the generation of ROS during inflammation [23, 36]. In addition, the constant exposure of the alveolar epithelial surface to high oxygen pressure makes the lung highly susceptible to free radical generation [34]. Öz and Ilhan evaluated the protective effect of melatonin against the toxic effects of DOX on lung tissue in rats [32].

Various attempts have been made to reduce DOX-induced toxicity. These include dosage optimization, synthesis and use of analogues. Moreover, a number of agents have been investigated as protective agents during DOX therapy [35]. A polyhydroxylated derivative of fullerene, named fullerenol (FLR) $C_{60}(OH)_{24}$ is being extensively studied due to its great potential as an antioxidant [9]. It is proposed that FLR may act as a free radical scavenger in biological systems, in xenobiotics as well as radioactive irradiations-induced oxidative stress. It has repeatedly demonstrated protective effects against the cytotoxicity of DOX in animal models [5, 9–11, 15–18, 30, 38, 39].

In the present study, we assessed the antioxidant status of the lungs of rats as well as the oxidative stress caused by DOX and investigated the potential protective effect of FLR $C_{60}(OH)_{24}$ on the lungs of rats with mammary carcinomas after a single dose of DOX.

Materials and Methods

Chemicals

FLR $C_{60}(OH)_{24}$ was obtained from the Faculty of the Science, University of Novi Sad (Novi Sad, Serbia). DOX and MNU (1-methyl-1-nitrosourea) were purchased from Pharmacia & Upjohn (Milan, Italy) and Sigma (Deisenhofen, Germany), respectively. MNU (14 mg/ml) and DOX (3 mg/ml) were dissolved in a sterilized and apyrogenic 0.9% NaCl solution, while FLR (20 mg/ml) was dissolved in a solution of 0.9% NaCl in water and Tween 80 (80/20, w/w). All solutions were made just before administration in a laminar flow cabin.

Animal treatment

In this investigation, 40 healthy adult male Sprague-Dawley outbred rats (3–4 weeks old) were used (Har-

Tab. 1. Experimental protocol

Group	Description	Experiment N	Surviving n	Administration – agent	DOX mg/kg	FLR mg/kg
I	Healthy control	8	8	*ip* – saline	/	/
II	Cancer control	8	8	*ip* – MNU* – saline	/	/
III	DOX	8	8	*ip* – MNU* – DOX	8	/
IV	FLR/DOX	8	8	*ip* – MNU* – FLR – DOX	8	100
V	FLR	8	8	*ip* – MNU* – FLR	/	100

* MNU – 50 mg/kg body weight (carcinogenicity was induced chemically) administered on the 50th and 113th day of age

lan, Italy). The animals were kept under standard laboratory conditions (22 ± 1°C; 70 ± 10% RH; 12 h light/dark cycle; 4 rats per cage). The animals had free access to food (Altromin, Germany) and water. The investigational protocol (Tab. 1) was approved by the National Animal Ethical Committee of the Republic of Slovenia (No. 3440-138/2006) and it met the guidelines of the European Convention for the protection of vertebrate animals for scientific purposes (ETS 123).

The rats (n = 40) were divided into five groups of eight and treatment was performed according to protocol shown in Table 1. On the 160th day of age, rats were treated with saline, DOX and/or FLR. Two days after administration, animals were sacrificed by CO_2.

The coefficient of the lung to body weight was calculated as the ratio of tissues (wet weight, mg) to body weight (g).

Histopathological examination

Lung tissues were harvested from the sacrificed animals. For histological analysis, the lobes were fixed in 10% buffered formalin, paraffin-embedded and sectioned at an average thickness of 5 μm. The specimens were stained with hematoxylin and eosin.

Biochemical analysis

Each lung was quickly removed from the sacrificed rat, placed in an ice-cold solution and adipose tissues were trimmed off. Afterwards, each organ was minced and homogenized in a Tris-buffer solution (pH 7.4; organ : buffer 1:10; w/w) and divided into two portions; one was used for malondialdehyde (MDA; Chromsystems Diagnostic, Munchen, Germany) determination, and the other was centrifuged at 13,000 × g for 20 min at 4°C (Beckman refrigerated, Ultracentrifuge). The supernatant was used for the assays of total protein concentration (TP; Sentinel Diagnostics, Milan, Italy), glutathione (GSH; Chromsystems Diagnostic, Munchen, Germany), glutathione peroxidase (GSH-Px; Ransel, Crumlin, UK), glutathione reductase (GR; Crumlin, UK), catalase (CAT) [2], lactate dehydrogenase (LDH; Chema Diagnostica, Jesi, Italy), total antioxidant status (TAS; Randox, Crumlin, UK) and superoxide dismutase (SOD; Ransod, Crumlin, UK).

Samples were stored at – 80°C before use.

Statistical analysis

Data were statistically analyzed by one-way analysis of variance (ANOVA) and LSD *post-hoc* test for multiple comparisons (SPSS 15.0 for Windows). Significance was set at $p < 0.05$.

Results

Effects of DOX and FLR treatment on the body weight of rats and lung coefficients

As shown in Table 2, the lung coefficient was significantly higher in the DOX group compared to the control and FLR groups ($p < 0.05$). The difference in the weight of the rats after sacrificing was not significant, with the exception of body weight differences between the FLR and healthy control group.

Tab. 2. Rat body weights, lung coefficients and the number of tumors after sacrificing (n = 8 per group)

Group	Body weight (g) after sacrificing	Coefficient of lung mg/g	Number of tumors n ± SD
I	271.2 ± 12.8	6.7 ± 0.7	/
II	262.5 ± 22.0	6.2 ± 0.8	4.5 ± 3.8
III	257.0 ± 15.8	6.8 ± 0.6[2a]	4.4 ± 2.3
IV	262.0 ± 13.8	6.3 ± 0.3	6.0 ± 2.7
V	251.5 ± 14.4[1a]	6.2 ± 0.5[3a]	3.0 ± 1.4

1,2,3 – Represents significant difference from the corresponding group ([a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$)

Effects of DOX and FLR treatment on lung histology

Histological changes in the lungs were evaluated as described in the Methods section. In the lung tissue, normal histological findings were seen in both control groups, as well as in the FLR pre-treated groups. Conversely, there were histological alterations in the DOX group. The acute change found in DOX group was subpleural edema, which correlates with the results for the coefficient of organs (data not shown).

Effects of DOX and FLR treatment on lung CAT, GR, GSH-Px, SOD, and LDH activities

CAT, GR, SOD, and LDH (Fig. 1) activities were found to be increased ($p < 0.05$) in the lung tissue of the animals in the DOX group over the all other groups, while GSH-Px had decreased ($p < 0.05$) activity compared with the control and FLR groups. There were no significant differences in enzyme activities in either control (healthy; cancer) or FLR (FLR/DOX; FLR) groups (Fig. 1).

Effects of DOX and FLR treatment on lung TAS, MDA, GSH, and GSSG levels

The results are summarized in Figures 2 and 3. TAS ($p < 0.001$) and MDA ($p < 0.001$ and $p < 0.01$) levels increased in both tissues in the DOX group in comparison with the control groups, while GSSG only increased relative to the cancer control group ($p < 0.001$). At the same time, pre-treatment with FLR provided marked normalization of MDA ($p < 0.001$),

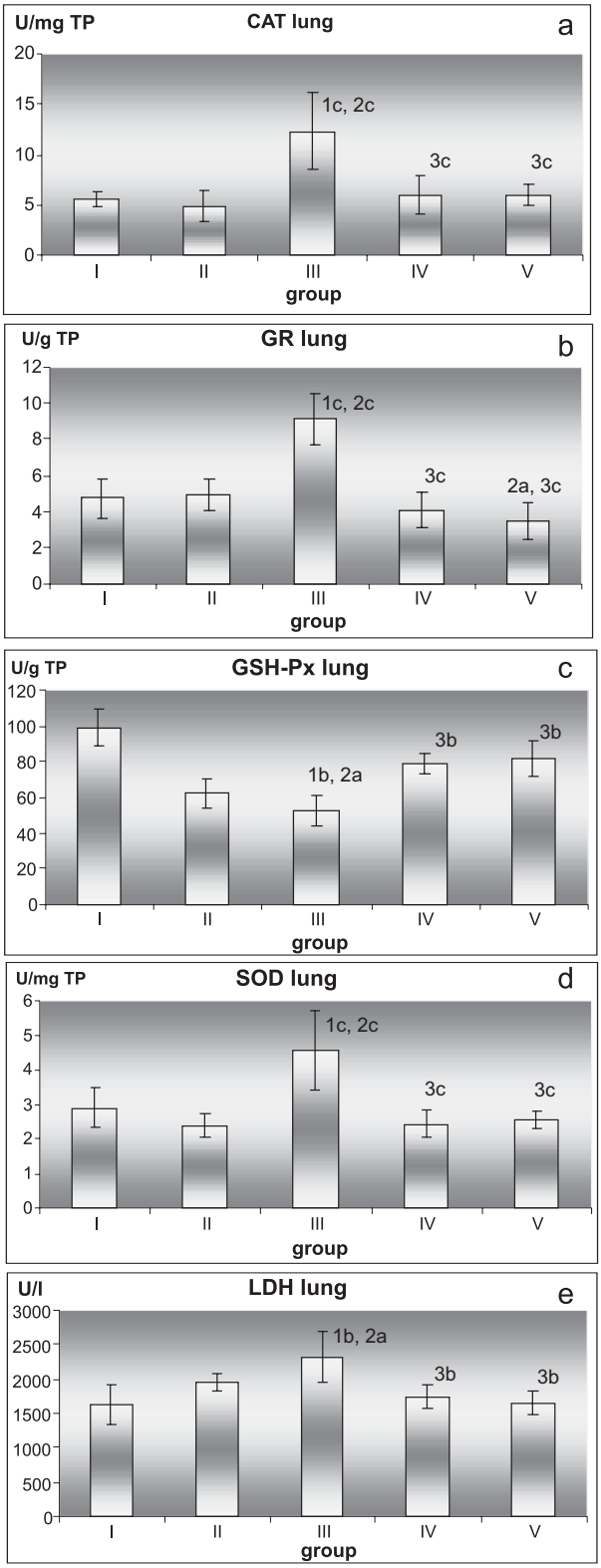

Fig. 1. Biochemical evaluation of (**a**) CAT, (**b**) GR, (**c**) GSH-Px, (**d**) SOD, and (**e**) LDH levels in lung samples (n = 8 per group). Significant difference from the corresponding group 1, 2, 3 ([a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$)

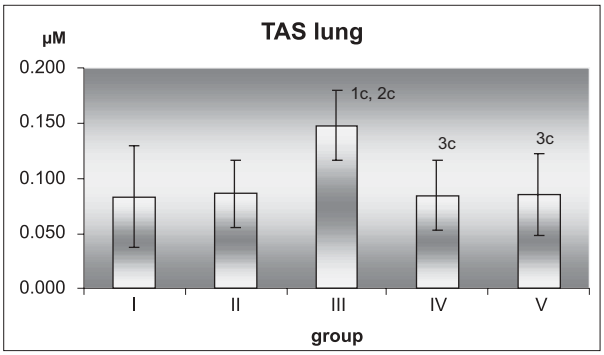

Fig. 2. Biochemical evaluation of TAS level in lung samples (n = 8 per group). Significant difference from the corresponding group 1, 2, 3 ([a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$)

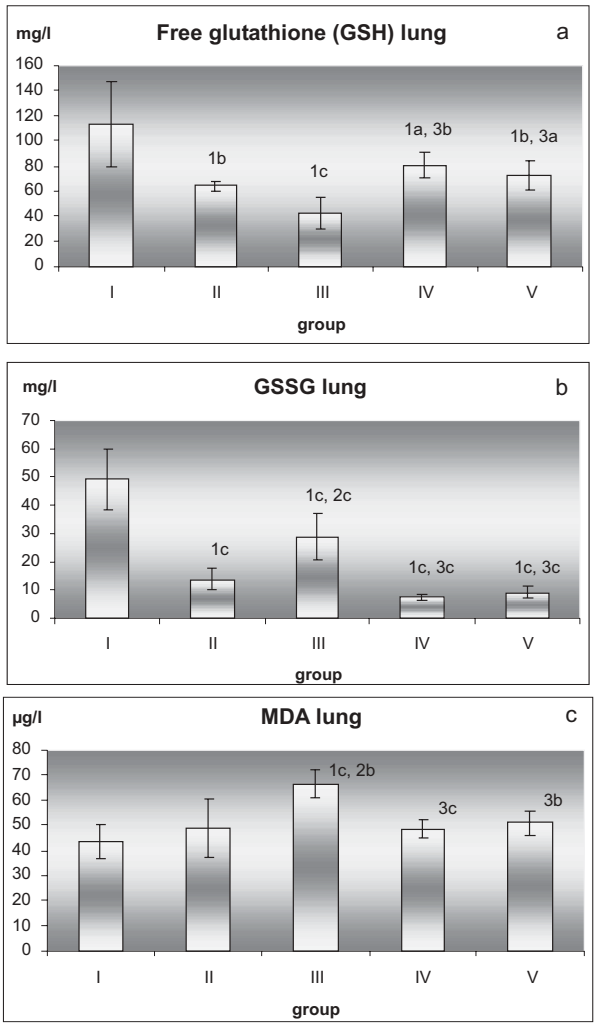

Fig. 3. Biochemical evaluation of (**a**) GSH, (**b**) GSSG, and (**c**) MDA levels in lung samples (n = 8 per group). Significant difference from the corresponding group 1, 2, 3 ([a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$)

GSH ($p < 0.01$), GSSG ($p < 0.001$) and TAS ($p < 0.001$) concentrations when compared to the group treated with DOX alone.

Discussion

It is widely accepted that oxidative stress and the production of free radicals are involved in DOX action, in relation to both anticancer effects and toxicity. Our investigation reveals that DOX treated rats showed significant changes in the lung coefficient in the acute phase, which might be due to the increased lipid peroxidation and ROS production, which in turn induces oxidative stress [32]. In relation to DOX pulmotoxicity, it is of value to remember that lung tissue is very sensitive to free radical damage due to, among other reasons, its increased oxidative metabolism and reduced antioxidant defense in comparison to other organs, such as the liver [25, 40]. The results of this study have confirmed that a single dose of DOX induces pulmotoxicity in rats in the acute phase. In the lungs of the DOX administered rats, MDA and GSSG levels were significantly elevated at the acute stages and supported the hypothesis that radicals play a major role in DOX pulmotoxicity [5, 10, 11, 15–18, 25, 29]. Minchin et al. [28] have reported pulmonary histopathological features induced by DOX treatment in *in situ* isolated dog lungs. Histopathologic investigation of the lungs from dogs receiving the highest concentrations of drug showed that necrosis of arterial endothelia and alveolar epithelia accompanied by periarterial edema, subpleural edema and emphysema of the lungs were the probable causes of acute animal mortality. The study confirmed that DOX produces dose-dependent injury to the pulmonary tissue. Our results are consistent with these reports in the relation to subpleural edema in DOX treated groups two days after administration and tissue injury (MDA, GSH, GSSG levels; Fig. 3).

Some recently published studies in man [4, 6, 13, 21, 22] have indicated that human lung resembles that of dog with respect to DOX uptake and retention, while the rate of drug accumulation is lower [27, 28]. Bleomycin and mitomycin are the chemotherapeutics most widely associated with lung damage, leading to chronic pulmonary fibrosis [25]. In combination with some other drugs commonly used in cancer therapy,

such as DOX, cyclophosphamide, vincristine, procarbazine, prednisone and gemcitabine, and bleomycin, can cause a variety of pulmonary toxicities ranging from temporary bronchospasm, which resolves spontaneously a few hours after the drug/s has/have been given, to acute interstitial pneumonitis, chronic pneumonitis and pulmonary fibrosis [6, 21].

LDH is the most used serum enzymatic marker for detection of tissue injury caused by DOX. An increase in the circulating levels of LDH in the DOX treated group was confirmed in our recently published paper for the same groups of rats [16]. A high LDH serum level is an index of myocardial infarction, renal failure, hepatitis, anemia, malignancies, and damaged skeletal muscles [16, 23]. Accordingly, we observed the LDH lung tissue level to find potential local cell injury. Minchin et al. [28] showed that even the lowest doses of DOX caused a significant increase in the reservoir of plasma LDH activity suggesting that some tissue damage had occurred. Our results for tissue LDH activity confirm that DOX causes lung tissue damage, while pre-treatment with FLR protects healthy lung cells.

The low GSH-Px activity could be directly explained by the low GSH content, since GSH is a substrate and cofactor of this enzyme (Figs. 1 and 3). Therefore, low GSH content implies low GSH-Px activity, which may produce increased oxidative stress propensity. These results for lung tissue are in agreement with the previously published manuscript from our research group and confirm the protective influence of FLR on DOX-induced pulmotoxicity. FLRs function as a free radical scavenger and antioxidant as shown in heart, liver and kidney tissue in rats with malignant neoplasm [15–17]. In all cases biochemical, hematological and histopatological parameters showed a significant decrease in cell injury when FLR was used as a pre-treatment agent during DOX therapy.

SOD activity in the lung significantly increased after 48 h of the DOX treatment, while the FLR groups had the same activity as both control groups (Fig. 1d). At this stage the increased dismutation of superoxide anions was probably due to the higher activity of the enzyme [24].

The increased CAT activity 2 days after the DOX injection suggests a compensatory response to oxidative stress due to a rise in endogenous H_2O_2 production. CAT activation has already been reported in most prior studies using DOX in doses of 8–15 mg/kg [19, 37], although more elevated doses (20 mg/kg) diminished or even had no effect on its activity [31]. Adachi et al. [1] have reported that the activities of SOD and CAT in the heart of mice were increased significantly by the *ip* administration of 15 mg/kg of DOX. In contrast, they found that these enzymes were unaffected by this dose of DOX in the liver and kidney. On the other hand, we reported a significant difference in SOD and CAT activity between the DOX group and other investigated groups in the heart [16], kidney [15], and liver [17] in rats with mammary carcinomas, after application of 8 mg/kg of DOX. After *ip* injection of FLR in a dose of 100 mg/kg (FLR/DOX; FLR), rats had the same CAT and SOD enzymatic activity as both control groups [15–17].

Very low antioxidant activity in lung tissue is also established by the TAS level (Fig. 2). The TAS test measures the total antioxidant effect of all defense systems in circulation (endogenous and exogenous). The antioxidant system protects tissue from the effects of the free radicals. A deficiency in any antioxidant will result in a reduction in the TAS. Therefore, high cell injury and low antioxidant activity is found in the DOX treated group and it is significant in comparison to the FLR investigated groups. Results for TAS confirm previously presented conclusions regarding the protective influence of FLR on lung, kidney, liver and heart tissue in DOX-induced toxicity [15–17].

Conclusion

In conclusion, oxidative damage to the lung induced by DOX may be one of the pathogenic factors of pulmonary dysfunction. It is possible that these deleterious effects may be limited by the use of FLR as pretreatment agent. FLR may be used to prevent pulmotoxicity during administration of DOX as a chemotherapeutic agent for malignancies. The key advantage of FLR in comparison to other known and investigated antioxidants or protectors is its dual purpose as radioprotector and organo-protector (heart, liver, kidney, and lung) during the anticancer therapy (radio- and chemo-). However, before a conclusive statement may be made on the potential usefulness of FLR as an adjunct to DOX therapy, there is a need for further studies to be undertaken, including human trials.

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