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Efficacy of amifostine in protection against doxorubicin-induced acute cardiotoxic effects in rats

Efikasnost amifostina u zaštiti od akutnih kardiotoksičnih efekata doksorubicina kod pacova

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Abstract

Background/Aim. Amifostine (AMI) is a broad-spectrum cytoprotector which protects against variety of radio- and chemotherapy-related toxicities without decreasing their antitumor action. The aim of the study was to investigate the potential protective effects of AMI against acute cardiotoxic effects of doxorubicin (DOX) in male Wistar rats. **Methods**. AMI (300 mg/kg *ip*) was given 30 min before DOX (6 mg/kg and 10mg/kg b.w., *iv*). The evaluation of DOX-induced cardiotoxic effects, as well as cardioprotective efficacy of AMI was performed 48 h after their administration by determining serum activities of enzymes known to be markers of cardiac damage (creatine kinase – CK, aspartate aminotransferase – AST, lactate dehydrogenase – LDH, and its isoenzyme α-hydroxybutirate dehydrogenase – α-HBDH), as well as the histopathological and ultrastructural analysis of the heart tissue. **Results**. AMI successfully prevented a significant increase in serum activity of CK, AST, LDH and α-HBDH in animals treated with DOX in the dose of 6 mg/kg (121.14 ± 18.37 *vs* 167.70 ± 44.24; 771.42 ± 161.99 *vs* 1057.00 ± 300.00; 3230.00 ± 1031.73 *vs* 4243.10 ± 904.06; 202.57 ± 42.46 *vs* 294.90 ± 80.20 UI/l, respectively), and ameliorated DOX-induced structural damage of the rat myocardium. Pretreatment with AMI in rats given 10 mg/kg DOX reduced the cardiac damage score (CDS) from 2.62 ± 0.51 to 1.62 ± 0.51, i.e. to the CDS value obtained with the lower dose of DOX (6 mg/kg). The ultrastructural analysis of the rat myocardium showed that AMI successfully protected the sarcolemma of cardiomyocytes and reduced mitochondria damage induced by DOX given in the dose of 6 mg/kg. Besides, capillaries were less morphologically changed and apoptosis of endothelial cells was extremely rare in AMI-protected animals. AMI itself did not cause any prominent changes in the examined parameters in comparison with the control rats. **Conclusion**. AMI provided a significant protection against DOX-induced acute cardiotoxic effects in rats. This finding implies its potential to be a successful cardioprotector in patients treated with DOX due to malignant diseases.

Key words:
amifostine; doxorubicin; heart; drug toxicity; cytoprotection; rats, wistar.

Apstrakt

Uvod/Cilj. Amifostin (AMI) je citoprotektor širokog spektra koji može da spreči ispoljavanje toksičnih efekata radio- i hemioterapije bez smanjenja njihovog antitumorskog dejstva. Cilj ove studije bio je ispitivanje efikasnosti AMI u zaštiti od akutnih kardiotoksičnih efekata citostatika doksorubicina (DOX) kod mužjaka Wistar pacova. **Metode**. AMI (300 mg/kg *ip*) davan je 30 min pre DOX (6 mg/kg i 10 mg/kg *iv*). Ispitivanje toksičnih efekata DOX, kao i kardioprotektivne efikasnosti AMI sprovedeno je 48 sati nakon njihove primene. U tu svrhu određivana je serumska aktivnost enzima, koji su poznati kao markeri oštećenja miokarda (kreatin kinaze – CK, aspartat aminotransferaze – AST, laktat dehidrogenaze – LDH, i njenog izoenzima α-hidroksibutirat dehidrogenaze – α-HBDH), i izvršena je patohistološka i ultrastrukturna analiza tkiva miokarda. **Rezultati**. Amifostin je uspešno sprečio značajno povećanje aktivnosti enzima CK, AST, LDH i α-HBDH u serumu životinja kojima je dat DOX u dozi od 6 mg/kg (121,14 ± 18,37 *vs* 167,70 ± 44,24; 771,42 ± 161,99 *vs* 1057,00 ± 300,00; 3230,00 ± 1031,73 *vs* 4243,10 ± 904,06; 202,57 ± 42,46 *vs* 294,90 ± 80,20 UI/l, redom), dok je kod pacova koji su dobijali DOX u dozi od 10 mg/kg smanjio skor oštećenja miokada sa 2,62 ± 0,51 na 1,62 ± 0,51, odnosno na vrednost skora dobijenu u grupi pacova sa nižom dozom

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DOX (6 mg/kg). Ultrastrukturna analiza tkiva miokarda pokazala je da je prethodna primena AMI kod pacova koji su dobijali DOX u dozi od 6 mg/kg uspešno zaštitila sarkolemu kardiomiocita i smanjila oštećenje mitohondrija i kapilara, kao i pojavu apoptoze endotelnih ćelija. Sam AMI nije izazvao nikakve značajnije promene u ispitivanim parametrima u poređenju sa intaktnim (kontrolnim) pacovima. **Zaključak**. Amifostin ispoljava značajan kardioprotektivni efekat kod pacova u ranom periodu posle primene pojedinačnih visokih doza DOX. Ovaj nalaz ukazuje na potencijal AMI da bude uspešan kardioprotektor i kod onkoloških bolesnika koji primaju DOX.

Ključne reči:
amifostin; doksorubicin; srce; lekovi, toksičnost; ćelija, zaštita; pacovi, wistar.

Introduction

Doxorubicin (DOX), anthracycline antibiotic, is an important antineoplastic agent due to its high antitumor efficacy in haematological, as well as in solid malignancies. However, adverse effects such as myelosuppresion and development of irreversible cardiotoxicity, manifested as a dilated cardiomiopathy leading to congestive heart failure, limit the use of DOX [1–4].

Although the molecular pathogenesis of DOX cardiotoxicity is still controversial, oxidative stress-based hypothesis involving intramyocardial production of reactive oxygen species (ROS) has gained the widest acceptance [5, 6]. Namely, drug toxicity may ensue through free-radical formation and a subsequent redox cycle with O_2, resulting in the generation of ROS, such as superoxide anions ($O_2^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$) and hydrogen peroxide. The tissues with less developed antioxidant defenses, such as the heart, are particularly susceptible to injury by DOX-induced oxygen radicals [7, 8]. Cell membrane lipids are the most common substrates for oxidative attack. Once initiated, peroxidation continues and has a progressive course that results in structural and functional changes in the heart tissue.

Since treating cardiac complications is very troublesome and expensive, a variety of efforts have been made to reduce this cardiotoxicity without compromising the antitumor activity of DOX [9–11]. One of them is the administration of the agent that would protect the myocardium from DOX toxicity. Considering the aforementioned mechanism of that toxicity, the approach based on the use of antioxidants, including free radical scavengers, seems to be rational.

Amifostine (AMI) is a broad-spectrum cytoprotective agent, with numerous preclinical and clinical studies suggesting protection against a variety of radio- and chemotherapy-related toxicities, including myelotoxicity, neurotoxicity and nephrotoxicity, without decreasing the antitumor action [12–16]. It is actually a prodrug that cannot protect tissues until dephosphorylated by alkaline phosphatase in the plasma membrane to the active metabolite, WR-1065. Once inside the cell, its protective effects appear to be mediated by scavenging free radicals, hydrogen donation, induction of cellular hypoxia, the liberation of endogenous nonprotein sulfhydrils (mainly glutathione) from their bond with cell proteins, the formation of mixed disulphides to protect normal cells etc. Until now not too many reports have been published concerning the prevention of DOX-induced cardiotoxicity by AMI [17–20].

The present investigation extended these studies. Serum activity of enzymes, known to be markers of compromised cardiomyocyte integrity and histological as well as ultrastructural analysis (UA) of the myocardial tissue were used to estimate the protective efficacy of AMI against DOX-induced acute cardiotoxic effects in rats. High, single doses of DOX, 6 mg/kg and 10 mg/kg b.w., were chosen by taking into account the cumulative DOX dose (450 mg/m^2 body surface or 11 mg/kg b.w.), known to produce potentially lethal cardiomiopathy in humans [21].

Methods

Experimental animals and the protocol

Adult male Wistar rats weighing 200 g to 250 g were used. The animals were housed in plastic cages, five animals per cage, under standard laboratory conditions (room temperature, 12/12 h light/dark cycle, free access to a standard rodent chow and water).

The animals were divided into 6 experimental groups of animals treated as follows:

The group I was the control one (saline, 1 ml/kg *iv*); the group II was treated with AMI (300 mg/kg *ip* 30 min before saline (1 ml/kg *iv*); the group III was treated with 6 mg/kg *iv* of DOX; the group IV was treated with 300 mg/kg *ip* of AMI 30 min before DOX (6 mg/kg *iv*); the group V was treated with 10 mg/kg *iv* of DOX and group the VI was treated with 300 mg/kg *ip* of AMI 30 min before DOX (10 mg/kg *iv*).

The study was based on the Guidelines for Animal Studies no 282-12/2002 (Ethics Committee of the Military Medical Academy, Belgrade, Serbia).

Drugs

AMI was synthesized in the Chemical Department of Military Technical Institute, Belgrade, by original procedure based on the method described by Piper et al. [22], as already published [23]. AMI was prepared for administration by dissolving the substance in sterilized and apyrogenic 0.9% NaCl solution, *ex tempore*. DOX was obtained from commercial sources (Adriblastina®, Hemofarm, Vršac in colaboration with Farmitalia Carlo Erba, Milan, Italy) and was dissolved in the water supplied in the original drug package, immediately prior to injection.

Evaluation of myocardial toxicity and its prevention

Since earlier pathohistological studies have revealed that structural damage of the rat heart occurs within 48 h

after application of 6 and 10 mg/kg of DOX [9, 24] we evaluated the efficacy of the pretreatment with AMI on DOX-induced cardiotoxicity within this period after their administration, according to the study protocol. Blood samples were collected from the caudal vein, just before sacrifice by decapitation under light ether anaesthesia. Hearts were removed rapidly and utilized for histopathological analysis (HA). Each experimental group consisted of 8 animals.

Enzyme assays

Blood samples were centrifuged at 3.000 rpm for 10 minutes. The serum activity of creatine phosphokinase (CK), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and its isoenzyme α-hydroxybutyrate dehydrogenase (α-HBDH) was determined on an autoanalyser Express 550 (Ciba Corning, Gilford Systems) using the test reagents produced by Randox firm (United Kingdom) and the procedures recommended by the manufacturer.

Histopathological analysis

The removed hearts were fixed in 10% formalin. Transmural tissue samples from the left and right ventricular free walls were embedded in paraffin blocks. Tissue samples 5-μm thick were stained with haematoxylin & eosin (HE) and heart sections were analyzed (20 x and 40x; Olympus-2 microscope; Tokyo, Japan). Grading of the cardiac tissue damages and calculating the cardiac damage score (CDS) were performed by using 0–3 scale as previously described [18], taking into account only myocytes showing cytoplasmic vacuolisation and/or myofibrillar loss. The grading system was as follows: 0 = no damage; 1 = < 5% myocytes damaged, 2 = 16%–25% myocytes damaged; 3 = > 35% myocytes damaged. Per eight hearts from each group were available, and per 5 sections from each heart were analyzed. All morphological examinations were performed by 3 independent observers as a blind study with no prior knowledge of the treatment given to the animals.

Tissue Preparation and Electron Microscopy

Another experiment, according to the same study protocol, has been done for electron microscopy examination. Immediately after the animals were sacrificed sections of the myocardial tissue were taken from the free wall of the left ventricle of each heart and small cubes of tissue were fixed in cold 4% glutaraldehyde with 0.1M sodium cacodylate buffer, at pH 7.2. After washing in the same buffer, the samples were postfixed with 1% osmium tetroxide, during 1 h, on + 4C° and contrasted by uranyl acetate during 24 h. The tissue was dehydrated in graded ethanol, transferred to propylene oxide and embedded in Epon. Sections were cut at 40 - 50 nm with a diamond knife on an LKB ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a Philips 201 C electron microscope. Each experimental group consisted of 5 animals.

Statistical analysis

The Student's *t*-test was used to asses differences in serum enzyme activity. Statistical evaluation of the difference in the severity of cardiac damage score among the various treatment groups was performed by using the Kruskal-Wallis rank test and Mann-Whitney U-test.

Results were considered significant when $p < 0.05$.

Commercial statistical software Stat for Windows, R.4.5., Stat Soft Inc., Tulsa, OK, USA, 1993, was used throughout the study.

Results

Effects of AMI on serum enzyme activity in DOX-treated rats

The assessment of cardiomyocytes integrity in the DOX-treated rats was done by determining the activity of AST, ALT, LDH and its isoenzyme α-HBDH in the serum. Serum activities of these enzymes were significantly increased in animals treated with both doses of DOX (6 and 10 mg/kg *iv*) comparing to those of the control group (Figure 1).

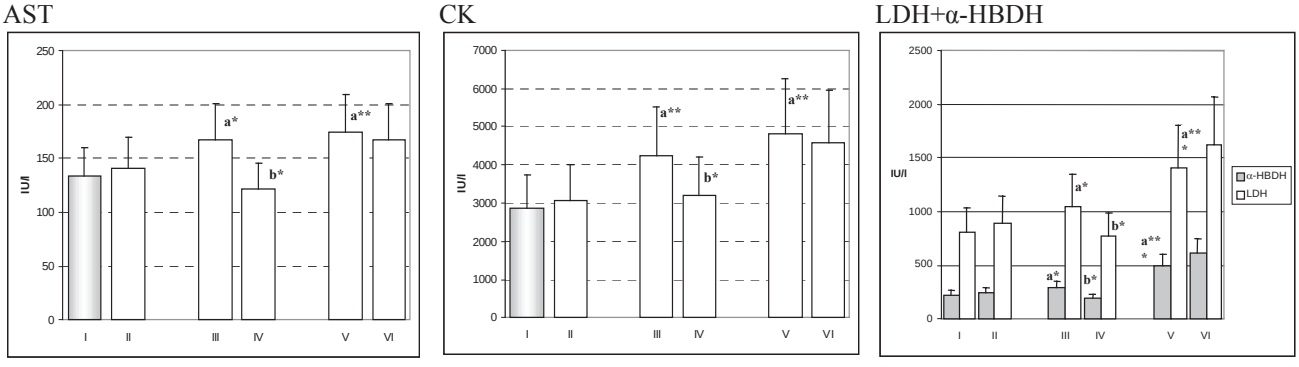

Fig. 1 – Influence of amifostine (AMI, 300 mg/kg *ip*) pretreatment on doxorubicin (DOX)-induced changes in aspartate aminotransferase (AST), creatine kinase (CK), lactate dehydrogenase (LDH) and α-hydroxybutyrate dehydrogenase (α-HBDH) serum activity in rats 48 h after their administration (AMI was given 30 min before *iv* injection of DOX, 6 mg/kg or 10 mg/kg)
I – the control (saline, 1 ml/kg *iv*); II – AMI; III – DOX (6); IV – AMI + DOX (6); V – DOX (10); VI – AMI + DOX (10)
a* , a, a*** – $p < 0.05$; $p < 0.01$; $p < 0.001$ *vs* I; b* - $p < 0.05$ *vs* III**

This increase was successfully prevented when animals were given AMI prior to being treated with DOX in a dose of 6 mg/kg. However, in the group of animals treated with 10 mg/kg of DOX, AMI failed to prevent DOX-induced increase of the serum activity of enzymes known to be markers of cardiomyocytes integrity damage.

On the other hand, AMI given before saline injection had no effect on the monitored parameters (Figure 1).

Effects of AMI on histopathological patterns of the hearts in DOX-treated rats

Light microscopic examination of the myocardium from DOX-treated rats in comparison with that of the control animals is shown in Figure 2. Histopathological analysis of the heart tissue of rats given both tested doses of DOX (6 mg/kg and 10 mg/kg) showed that most of the cardiac muscle cells were regularly arranged. However, in animals treated with 6 mg/kg of DOX a certain number of cardiomyocytes with fine granular cytoplasm, without clearly noticeable nuclei, was detected, some of which had small vacuoles and/or pale appearance of the cytoplasm. In animals pretreated with AMI just a small number of myocytes with fine granular cytoplasm was seen differing from surrounding normal myocardial tissue. The appearance of numerous vacuoles and segmental loss of normal tissue structure was seen in rats treated with 10 mg/kg DOX, while in animals pretreated with AMI more preserved myocardial structure was visible, with less extensive vacuolization of cardiomyocytes (Figure 2). Grading cardiac tissue damages by 0–3 scale in rats, treated with DOX in single doses of 6 and 10 mg/kg, revealed CDS of 1.62 ± 0.51 and 2.62 ± 0.51, respectively. The differences between the control and DOX treated groups were statistically significant (Table 1). In the group of rats treated with 6 mg/kg of DOX which had previously received AMI, myocyte alterations were significantly less severe than those observed in animals without pretreatment ($p < 0.01$). Pretreatment with AMI in rats given DOX in a dose of 10 mg/kg reduced CDS to the value obtained in the group of rats treated with 6 mg/kg of DOX (Table 1).

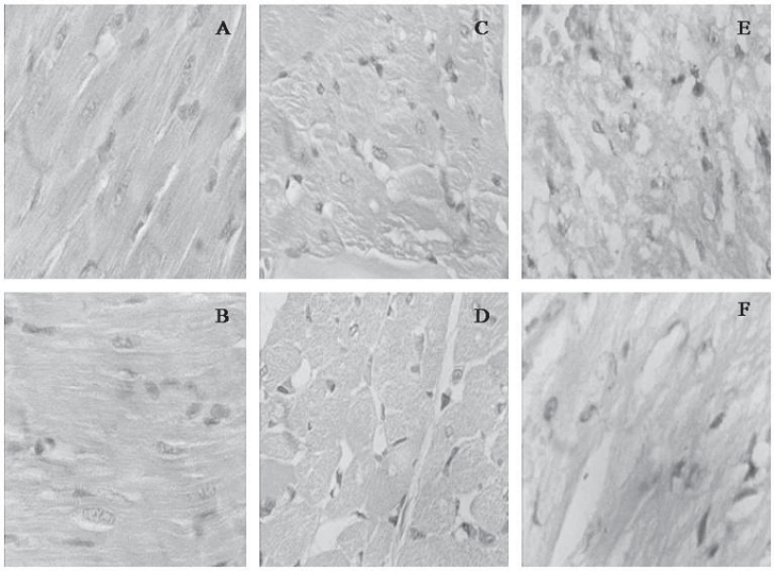

Fig. 2 – Light microscopy of the heart sections: (A) control group – myocardium of normal morphology, (B) group treated with amifostine (AMI) – no histological lesions found (H&E, ×40), (C) group treated with doxorubicin (DOX) 6 mg/kg – small number of myocites with discrete vacuolization, (D) group treated with AMI + DOX 6 mg/kg – a small number of cardiomyocytes with fine granular cytoplasm (H&E, ×20), (E) group treated with DOX 10 mg/kg – appearance of numerous vacuoles and segmental loss of normal tissue structure, (F) group treated with AMI + DOX 10 mg/kg – less extensive vacuolization of cardiomyocytes with more preserved myocardial structure (H&E, ×40)

Table 1

The influence of amifostine on cardiac damage scores (CDS) in rats treated with doxorubicin

Treatment (mg/kg)*	Cardiac damage score (CDS)** (8 hearts x 5 section)				Mean CDS ± SD
	0	1	2	3	
Control (saline, 1 ml/kg *iv*)	30	10	0	0	0.25 ± 0.46
AMI (300)	30	10	0	0	0.25 ± 0.46
DOX (6)	0	15	25	0	1.62 ± 0.51 [a]
AMI (300) + DOX (6)	25	10	5	0	0.50 ± 0.75 [b]
DOX (10)	0	0	15	25	2.62 ± 0.51 [a]
AMI (300) + DOX (10)	0	15	25	0	1.62 ± 0.51 [a b]

***Amifostine (AMI) was administered *ip* 30 min before doxorubicin (DOX) given *iv*; ** CDS: 0 – no damage; 1 – < 5% myocytes damaged; 2–16% to 25% myocytes damaged ; 3 – > 35% myocytes damaged; † Statistical evaluation was performed using Kruskal-Wallis test: [a] $p < 0.001$ *vs* control; Mann-Whitney U test: [b] $p < 0.01$ *vs* corresponding DOX group**

In animals sacrificed 48 h after giving AMI 30 min before saline (1 ml/kg *iv*) no any pathological changes were found, nor CDS was significantly different from that of the control group.

Effects of AMI on ultrastructural alterations of the hearts in DOX-treated rats

Ultrastructural analysis (UA) of the heart sections of rats treated with DOX in a dose of 6 mg/kg showed prominent alterations comparing to those of the control rats (Figure 3a). Cardiomyocytes were transparent, with preserved volume. Nuclei of the cardiomyocytes had an altered shape, with shallow invagination of nucleus membrane and enlarged perinuclear spaces. Mitochondria were numerous, hydropically degenerated with enlarged volume and light matrix. Their cristae were moved to periphery (Figure 3b). Sarcolemma of some cardiomyocytes were locally lysed and mitochondria could be seen out of the cell, in intercellular spaces. Endothelial cells in the capillaries between cardiomyocytes showed changes that could be described as the ones characteristic for programmed cell death – apoptosis. These cells became very thin, with condensed, dark cytoplasm and heavily condensed chromatin filling the majority of caryoplasma. (Figure 3c). In some biopsies rupture of capillary walls could be seen.

In the animals which received AMI before DOX injected in the same dose (6 mg/kg *iv*), structural changes were prominently less expressed, with no lysis of cardiomyocytes sarcolemma. Nuclei of myocytes were, most often, like those in the control animals, while the mitochondria damage was less prominent (Figure 3d). Capillaries were less morphologically changed and apoptosis of endothelial cells was extremely rare.

The application of AMI itself, without DOX, led to discrete changes of the cardiomyocytes comparing to the control animals. Shallow invagination of the nucleus membrane and marginal condensation of heterochromatin were most prominent. Mitochondria with lamellar cristae predominated in this group of animals.

Discussion

The results of this study showed that the serum activity of CK, AST, LDH and its isoenzyme α-HBDH, as the most characteristic marker for cardiac damage, was significantly increased in the animal groups treated with both doses of DOX comparing to the control rats, in a dose- dependant way. The elevation of serum concentrations of examined enzymes is a well-known quantitative index of compromised cellular integrity, and is also considered to be a good indicator of myocardial damage by DOX [25–27]. Formation of free radicals and peroxidation of lipids of cardiomyocyte membranes, including sarcolemma, caused by DOX, is thought to be followed by membrane permeability and other changes of membrane functions. Our findings are in accordance with the results of other authors who showed that increased serum activity of CK and LDH was detected in the period lasting between a few hours and 4 days after the administration of DOX doses ranging from 10 to 20 mg/kg, with the peak at day 2 [10, 27, 28]. It was considered that a damaged sarcolemma enables the enzymes to pass out of the cell, thus accounting for their prominent increase in the serum. This was actually

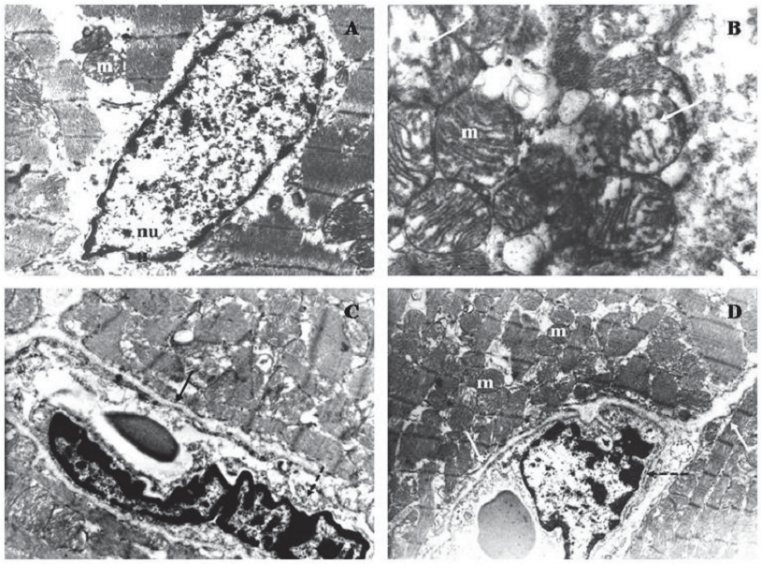

Fig. 3 – Electron micrograph of myocardium from: (A) control group of animals – control heart demonstrating normal peripheral distribution of nuclear chromatin (nu), sarcomeres, and mitochondria (original magnification ×36,000) (B) group of animals treated with doxorubicin (DOX) 6 mg/kg – mitochondria (m) hydropically degenerated with enlarged volume and light matrix (→). Cristae are moved to periphery (original magnification ×67,500). (C) group of animals treated with DOX 6 mg/kg – prominently thin capillary wall (→); endothelial cell nucleus irregularly shaped with increased quantity of heterochromatin (⇒) (original magnification ×23,850) (D) group of animals treated with amifostine (AMI) + DOX 6 mg/kg – mitochondria like in control animals, sarcolemma is preserved (→), capillary endothelial cell nucleus with marginally distributed heterochromatin (⇒) (original magnification ×30,000)
Note: AMI (300 mg/kg *ip*) was given 30 min before DOX

confirmed in our experiment in which the UA of the heart sections of the rats treated with 6 mg/kg of DOX showed that the sarcolemma of some cardiomyocytes was locally lysed and mitochondria could be seen out of the cell, *ie* in intercellular spaces.

On the other hand, HA revealed, taking into account only myocytes showing cytoplasmic vacuolization and/or myofibrillar loss, CDS of 1.62 ± 0.51 and 2.62 ± 0.51 in rats treated with 6 mg/kg and 10 mg/kg of DOX, respectively. The differences between the control and DOX-treated groups were dose-dependent and statistically significant. The myocardial cellular alterations associated with the administration of DOX in our experiments were similar to those reported in previous experimental studies [9, 24, 28, 29]. The affected myocytes displayed two characteristic light microscopic changes: cytoplasmic vacuolization and/or myofibrillar loss. The more myocytes showed these changes, the more pronounced the lesions became. UA of the rat heart 48 h after administration of 6 mg/kg of DOX revealed cardiomyocyte alterations described as oncosis. In parallel with the preserved volume and marginally condensed heterochromatin these cells had hydropically degenerated mitochondria with the light matrix and cristae moved to periphery. This was in accordance with the results of other authors who showed that the earliest and most often changes in the rat heart after application of DOX high doses were cellular oedema and swelling of the mitochondria in cardiomyocytes [28]. It is widely accepted that oncosis, as a type of prelethal changes, is characterized by the loss of cell volume control, typically resulting from adenosine triphosphate (ATP) deficiency and subsequent failure of Na^+-K^+ATPase at the plasmalemma, early clumping of nuclear chromatin, swelling of the mitochondria and dilatation of the endoplasmic reticulum (ER) and Golgi components [30–32]. On the other hand, apoptosis is characterized by cell shrinkage, accompanied by marked cell shape changes with multiple cytoplasmic protrusions and nuclear irregularities with intense chromatin clumping. The cytosol is electron-dense though some ER dilatation and mitochondrial condensation occur. Biochemically, there are both maintenance of ATP in the cell and the increased level of Ca^{2+}. In our experiments apoptotic cardiomyocytes were not observed. That can be explained by the fact that some special stainings, including TUNEL assay, are necessary for their detection. Also, Arola et al. [29] showed that 2 days after *ip* injection of DOX in the dose of 5 mg/kg only 0.033% of cardiomyocytes had TUNEL-positive nuclei (comparing with 0.0065% in control). The current understanding of molecular mechanisms underlying DOX-induced cardiomyocyte type of death, both apoptosis and necrosis, still imply excessive production of ROS. However, it is considered that predominant mechanism of cell death is determined by DOX dosage. Namely, low-dose DOX exposure induced apoptosis whereas high-dose exposure primarily induced oncosis of myocytes [5, 6, 33]. The latter corresponds to our experimental conditions. On the other hand, UA revealed some capillary endothelial cells with morphological changes characterizing apoptosis, in accordance with the results of other authors [34–36].

AMI successfully prevented significant increase of serum activity of all the examined enzymes in animals treated with DOX in a dose of 6 mg/kg. In AMI protected animals myocyte alterations were significantly less severe than those observed in animals without pretreatment. Moreover, the pretreatment with AMI in rats receiving higher dose of DOX (10 mg/kg) reduced CDS to the value obtained in the group of unprotected rats given 6 mg/kg of DOX. UA actually showed that the pretreatment with AMI in rats receiving 6 mg/kg of DOX protected the sarcolemma of cardiomyocytes, and significantly reduced mitochondria damage. Moreover, in the protected rats myocardial capillaries were less morphologically changed and apoptosis of endothelial cells was extremely rare. AMI itself did not cause any changes in all of the examined parameters in comparison with the control rats. Previous *in vitro* studies demonstrated that WR-1065, the active metabolite of AMI, was able to scavenge $OH^{\cdot}$ and $O_2^{\cdot -}$, including DOX-derived $O_2^{\cdot -}$ generated by NADH respiration of heart mitochondria particles [37]. Many studies still support the hypothesis that mitochondria are a primary target of DOX-induced oxidative stress. The fact that typical mitochondrial density per cell unit volume ranges from 25% to 35% in cardiomyocytes may partially explain why DOX is selectively toxic to the heart [38, 39]. AMI is a negative charged thiol which accumulates within the mitochondria and around DNA. These facts explain higher protective potential of AMI compared with that of neutral or positive charged thiols, taking into account some studies using perfused rat hearts which have shown that DOX is localized primarily arround the nucleus and within cell mitochondria [39, 40]. Also, both AMI and WR-1065 significantly reduce DOX-induced heart cell toxicity, measured by ATP content, normalised to the total cellular protein [37]. That can also be explained by their effective protection of mitochondria, as in our study, since oxidative phosphorylation is one of the functions of this organelae which provides a substantial portion of the ATP needed to meet energy demands of the heart. On the other hand, several lines of evidence suggest that AMI is presumably modified by membrane-bound alkaline phosphatase which is highly expressed in the endothelium and transferred into WR-1065. Then, WR-1065 quickly penetrates into cells, and acts as free-radical scavenger protecting them from oxidative damage [13, 14, 41]. Potent protective effects of AMI pretreatment in the model of pulmonary endothelial cell barrier dysfunction *in vitro* were shown. Owing to AMI the attenuation of oxidative stress, NF-κB inflammatory cascade and disruption of endothelial cell adhesions leads to the preservation of endothelial cell monolayer integrity [42]. On the other hand, marked elevation of the expression of antioxidant enzyme manganese superoxide dismutase (MnSOD) gene in human microvascular endothelial cells following their exposure to a WR-1065 can result in elevated resistance to the cytotoxic effects of ionizing radiation. Namely, MnSOD is nuclear-encoded mitochondrial enzyme that scavenges $O_2^{\cdot -}$ in mitochondrial matrix, and has been shown to be highly protective against radiation-induced ROS [43]. Based on the current data, the present authors speculate that succesful AMI protection of DOX-induced damage of heart capillaries,

whose endothelium as a rich source of oxidants contributes a lot to the oxidant-rich environment at that locus in this model, may be mediated by AMI antioxidant properties resulting in downregulation of oxidative stress and redox-sensitive signalling cascades. Bolman et al. [19, 44] have shown that AMI significantly decreases DOX-induced lipid peroxidation (evaluated by malondialdehyde level) and increases the levels of reduced gluthatione (GSH) and catalase activity in the hearts of rats treated by high doses of DOX. According to Luo et al. [26], after the application of DOX, ROS by inducing lipid peroxidation produce cytotoxic aldehydes resulting in inflammatory reactions. This eventually leads to increased synthesis of cytokines, infiltration of mononuclear cells and death of cardiomyocytes. In accordance with this, in our previous experiments the presence of mononuclear cells and fibroblasts was decreased in AMI-protected rats and necrotic myocytes were rare compared with DOX-only treated group [18]. However, the high dose of DOX was a cumulative one, given as a multiple, low, unitary dose regimen, with AMI always preceding DOX. According to that, our own results [18], as well as some others' [45, 46] support the statement that acute and chronic cardiac toxicity of DOX share the same mechanism, implying that chronic toxicity arises from repeated episodes of acute exposure which induces a cumulative damage. However, since single doses of DOX used in this experiment were very high, AMI might produce its cardioprotective effect by some other mechanisms, besides the antioxidative one. For example, it has recently been shown that AMI, given in doses similar to that used in this experiment, produced a strong anti-inflammatory activity [42, 47, 48] that might additionally offer protection against DOX-induced cardiac damage. However, further investigations are needed to confirm this hypothesis.

Conclusion

In summary, the present study demonstrates the potent protective effects of AMI pretreatment against acute cardiotoxic effects of DOX given in single high doses in rats. The obtained results imply the potential of AMI to be a successful cardioprotector in patients treated by DOX due to malignant diseases.

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