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Evaluation of diphenyleneiodonium influence on cardiac morphology and selected redox equilibrium markers in rats treated with doxorubicin

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Summary

The aim of the study was to evaluate the effect of diphenyleneiodonium sulfate (DPI) on cardiac morphology and oxidative stress secondary to doxorubicin administration. Rats were intraperitoneally injected with doxorubicin (1.5 mg/kg) once a week for ten weeks (DOX group). In each case, DPI was administered subcutaneously at two single doses (0.25 or 1 mg/kg), 24 h and 4 h before and 24 h after doxorubicin injection (DPI+DOX groups). Necropsy was performed three weeks after the completion of doxorubicin treatment. DPI significantly reduced the cardiac NADPH level and, at the higher dose, normalized cardiac lipid peroxidation altered by doxorubicin. There were no significant changes in the levels of cardiac NADH, glutathione, nor differences in plasma cardiac troponin I, fatty acid binding protein, and B-type natriuretic peptide, between the DOX and DPI+DOX groups. No cardiomyocyte necrosis was observed in biochemical and morphological examinations. However, DPI highly augments other cardiac morphological changes caused by doxorubicin.

Keywords: doxorubicin, iodonium sulfate, flavin enzymes, oxidative stress, redox balance

Doxorubicin (DOX) is a highly effective chemotherapeutic agent, but its use is associated with a risk of life-threatening delayed cardiomyopathy. Numerous attempts have been made to alleviate the cardiotoxic effect of DOX. Many antioxidants and free radical scavengers were extremely effective under laboratory conditions (14, 25, 30, 34). However, none of them showed significant benefits in clinical practice. The latest studies indicate that some drugs, although not introduced into clinical practice, were effective in clinical trials (53). Moreover, the protective effect of telmisartan is partially attributed to its intrinsic antioxidant activity (5). In view of the fact that the strategy based on the scavenging of reactive oxygen species (ROS) is not effective, in this study the authors proposed another approach, based on the prevention of ROS production. This concept is supported by the fact that dexrazoxane, the only drug approved by American Food and Drug Administration (FDA) for prevention of DOX-related cardiomyopathy, acts by preventing ROS generation (19, 42). In this study, diphenyleneiodonium sulfate (DPI) was used as an inhibitor of enzymes triggering ROS synthesis in rats receiving DOX (Fig. 1).

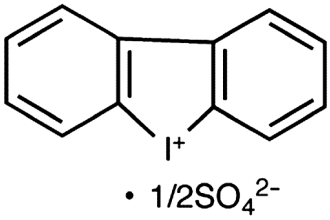

Fig. 1. Structure of diphenyleneiodonium sulfide

The prevalent hypothesis explaining DOX-related delayed cardiotoxicity ascribes the dominant role to the oxidative stress linked to redox-cycling of the drug (51). The redox-cycling begins with one-electron enzymatic reduction, with the formation of the DOX radical (DOX*), and then an electron from DOX is spontaneously taken by oxygen (O_2) (1). This results in the production of anion superoxide (O_2*-), while DOX* is transformed into DOX, ready to take the next electron (13). The one-electron reduction is catalyzed by NADPH and NADH-dependent enzymes, e.g. NADPH cytochrome P450 reductase (P450R) (11), nitric oxide synthase (NOS) (32, 35, 50), xanthine oxidase (OX) (52), and mitochondrial complex I (10). Recently, it has been demonstrated that NADPH oxidase plays an important role in DOX-related cardiomyocyte disorders (9, 17). These enzymes are also inhibited by the DPI compound. Table 1 presents DPI inhibitory activity towards enzymes responsible for DOX cardiotoxicity and the role of these enzymes in DOX toxic activation. These data demonstrate that the DPI inhibition of all enumerated flavin enzymes may play an important role in tempering DOX-related cardiotoxicity. NADPH oxidase possessing the lowest Ki (inhibition constant) is the enzyme most susceptible to DPI. Among all NOS isoenzymes, isoform III is characterized by the highest efficiency (the lowest Michaelis constant value) of DOX univalent reduction. However, DOX may upregulate NOS I synthesis, increasing DOX bioactivation several times (31). Additionally, NADH oxidase also attracts some attention, as its inhibition may stop the proliferation of certain neoplastic cells (10-22). DPI is a model compound inhibiting flavoenzymes, but its administration results in various side effects (18, 20, 21). When the cardioprotective effect of flavin enzyme inhibition has been confirmed, new, more specific and less toxic compounds should also be evaluated. The aim of the study was to evaluate the effect of DPI on cardiac morphology and oxidative stress secondary to doxorubicin administration.

Tab. 1. Magnitude of flavin enzyme inhibition by DPI and the effectiveness of these enzymes in doxorubicin reduction

Flavin enzyme	Inhibition by DPI	Enzyme-related mechanism of DOX
NADPH cytochrome P450 oxidoreductase	Ki: 2.8 μm (48)	Km (μM DOX): 112 (11)
Nitrix-oxide synthase	IC_{50}: 50 μM (45)	Km (μM DOX): 138 (NOSI) (16) Km (μM DOX): 210 (NOSII) (16) Km (μM DOX): 40 (NOSIII) (16) Km (μM DOX): 5 (NOS III) (50)
NADPH oxidase	Ki: 0.1 μm (18) Ki: 5.6 μM (36)	Cardiac remodeling (55) Increases superoxide formation (9) Cardiac myocyte apoptosis (17)
NADH dehydrogenase complex	Strong inhibition (26) Ki: 23 nmol/mg of protein (38)	Km (μM DOX): 454 (11)
Xanthine oxidase	–	Significant formation of superoxide (11)

Explanations: Ki – inhibition constant; Km – Michaelis constant

Material and methods

Animals and treatment. The experimental protocol was approved by the Local Bioethical Committee at the Medical University, Lublin (#40/2006). The study was conducted on sexually mature albino rats of the Wistar CRL: (WI)WUBR strain obtained from a commercial breeder (Warsaw-Rembertów, Poland). Animals with the initial body weight of 160-195 g were maintained under stable conditions at 22°C with a 12 h light/dark cycle and given standardized granulated fodder LSM (AGROPOL, Poland). The rats were intraperitoneally exposed to doxorubicin (DOX; Ebewe, Austria) and subcutaneously to diphenyleneiodonium sulfate (DPI; Toronto Research Chemicals TRC Inc, Canada). The animals were randomly divided into six groups (n = 8): DOX – doxorubicin 1.5 mg/kg body weight (BW); 1 DPI+DOX – diphenyleneiodonium sulfate 1.0 mg/kg BW and doxorubicin 1.5 mg/kg BW; 0.25 DPI+DOX – diphenyleneiodonium sulfate 0.25 mg/kg BW and doxorubicin 1.5 mg/kg BW; 1 DPI – diphenyleneiodonium sulfate 1.0 mg/kg BW; 0.25 DPI – diphenyleneiodonium sulfate 0.25 mg/kg BW, and the untreated control. Doxorubicin at a dose of 1.5 mg/kg BW was administered once a week for ten weeks. In each case, DPI was injected 24 and 4 h before and 24 h after doxorubicin administration. Three weeks after the last dose of doxorubicin the animals were sacrificed, and their blood and hearts were collected during autopsy. The heart was washed with 20 ml of saline, then sectioned along the interventricular and coronal grooves. The left ventricular wall was placed in liquid nitrogen and stored at –75°C until biochemical determinations. The right ventricular wall samples were fixed in 10% buffered formalin and routinely histologically processed.

The evaluation of serum biochemical parameters and tissue markers for redox imbalance. All parameters were determined with a microplate reader (BIO-TEK XS PowerWave, USA). Cardiac troponin I (cTnI; Life Diagnostics, #2010-2-HS, USA,), heart-fatty acid binding protein (H-FABP; Life Diagnostics, #2310-2-HS, USA), and B-type natriuretic peptide (BNP; Assay Pro, #ERB1202-1, USA) levels were assessed in rat serum with ELISA commercial kits according to the manufacturer's instruction. NADPH and NADH levels were determined by the spectrophotometric method described in the commercial kit (Bio Vision, USA, #K347-100, and #K337-100, respectively). The frozen cardiac samples (~20 mg) were homogenized in an extraction buffer provided by the manufacturer and then incubated at 60°C to decompose NADP or NAD particles. Final readings were made at 450 nm. The total glutathione in the heart homogenate supernatant was measured with BIOXYTECH GSH-420 (OxisResearchTM, #21023, USA). In the first step of this procedure, oxidized glutathione was reduced, and then total glutathione was determined in its reduced form (GSH). After a reaction with chromogen (1-methyl-4chloro-7-trifluoromethylquinolinium in HCl), a colored product was determined at 420 nm. The evaluation

of lipid peroxidation products was based on malondialdehyde (MDA) levels in cardiac homogenates. A commercial kit TBARS (Cayman, #10009055 USA) was used for the assessment. The method is based on the reaction between MDA and thiobarbituric acid at 100°C and low pH.

Preparation of slides for histological evaluation. Four-µm thick histological slides obtained from paraffin blocks were routinely processed and stained with hematoxylin and eosin (H&E) or by the van Gieson method. All slides were evaluated under a light microscope (Olympus BX45; Tokyo, Japan) by experienced pathologists, with all treatment groups blinded. The presence of histological features was assessed on a three-grade scale, according to which "no change," "low intensity," and "moderate intensity" were assigned „0," „+," and „++," respectively.

Statistical analysis. The data obtained were expressed as mean ± SD and statistically analyzed with the Statistica 5.0 software. Continuous data were compared between the experimental groups by the Kolmogorov-Smirnov test. The statistical significance of differences between the control and other groups was evaluated by either the t-Student test or the U Mann-Whitney test, and group to group comparisons were made by the one-way ANOVA. The value of $p \leq 0.05$ was assumed as statistically significant.

Tab. 2. Incidence (%) of common histopathological changes in the myocardium

Group	Interstitial fibrosis		Perivascular fibrosis		Interstitial edema		Cardiomyocyte edema		Cytoplasm vacuolization		Cell infiltration		Cardiomyocyte hypercontraction	
	+	++	+	++	+	++	+	++	+	++	+	++	+	++
Control	22.3	–	44.5	–	11.2	–	–	–	11.2	–	33.4	–	11.2	–
DOX	40.0	–	80.0	–	60.0	–	–	–	60.0	–	100.0	–	60.0	–
1 DPI	50.0	–	100.0	–	62.5	–	25.0	–	37.5	–	87.5	–	50.0	–
0.25 DPI	50.0	–	75.0	–	62.5	–	50.0	–	25.0	–	62.5	–	12.5	–
1 DPI + DOX	100.0	–	100.0	–	77.7	22.3	44.5	–	55.5	–	88.8	–	22.3	–
0.25 DPI + DOX	62.5	25.0	75.0	25.0	62.5	12.5	25.0	–	87.5	–	62.5	25.0	12.5	–

Explanations: Incidence was calculated in relation to all animals in each experimental and control group

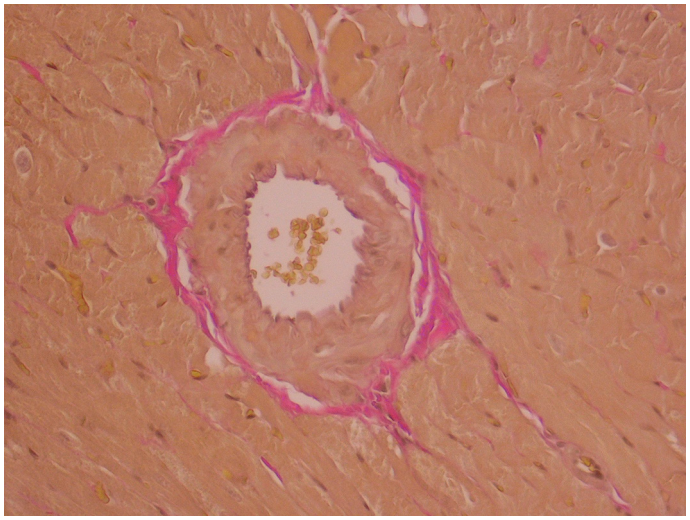

Fig. 2a. Perivascular fibrosis in the DOX group (van Gieson; objective mag. ×20)

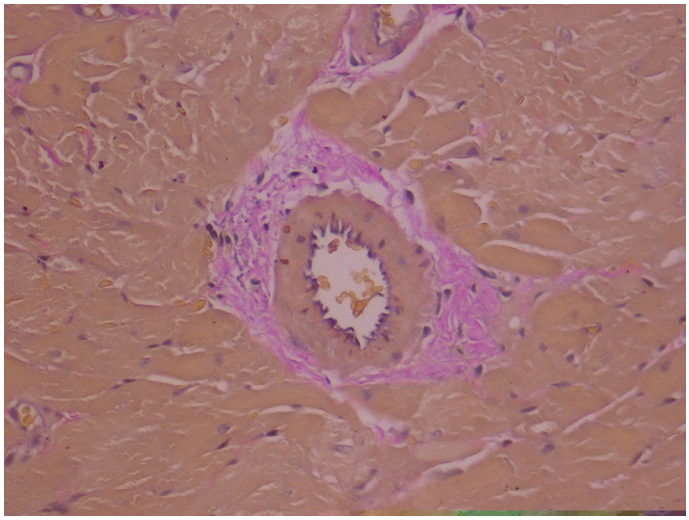

Fig. 2b. Perivascular fibrosis in the 1 DPI+DOX group (van Gieson; objective mag. ×20)

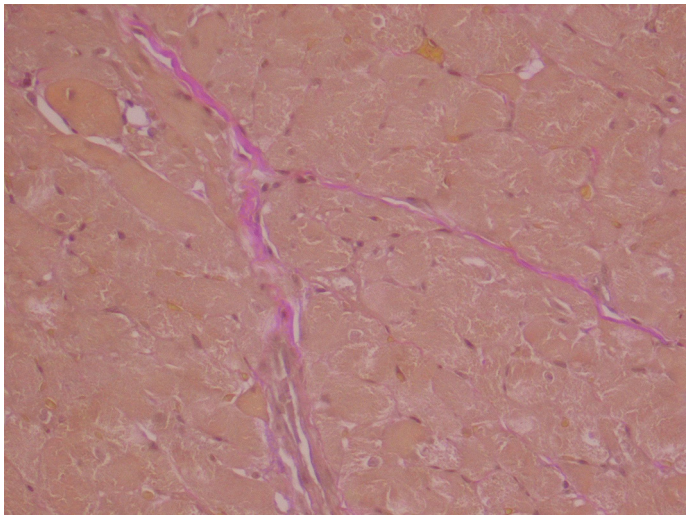

Fig. 2c. Interstitial fibrosis in the 1 DPI+DOX group (van Gieson; objective mag. ×20)

Results and discussion

The drugs were well tolerated, and no deaths occurred. Histological cardiac abnormalities were mostly limited to local changes of low intensity (+) (Tab. 2). Only in the DPI + DOX groups a moderate intensity (++) of pathological changes was observed. The incidence of interstitial and perivascular fibrosis, as well as interstitial edema, was higher in the DOX and DPI groups than it was in the control group (Fig. 2, 3). Moreover, interstitial edema was observed more often in the groups exposed exclusively to DOX

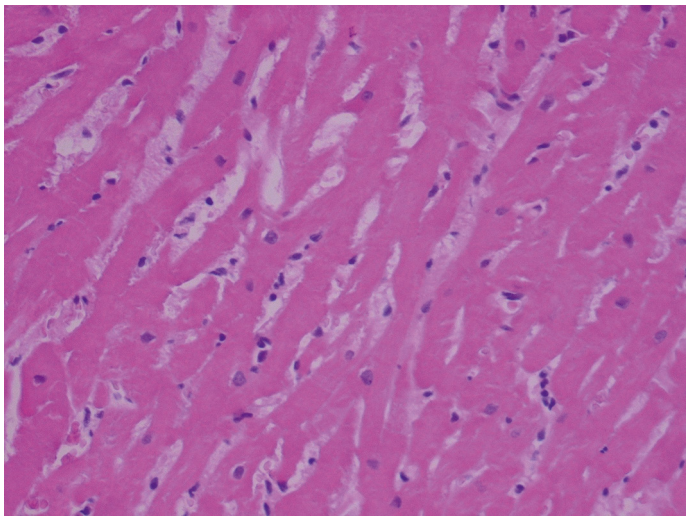

Fig. 3a. Connective tissue edema in the 1 DPI group (H&E, objective mag. ×10)

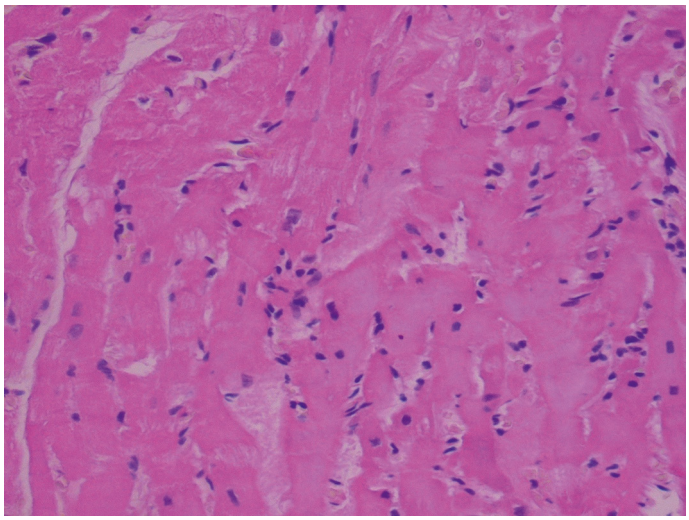

Fig. 3b. Connective tissue edema in the 1 DPI+DOX group (H&E, objective mag. ×10)

or DPI than in the control group. DPI induced fibrosis, edema, and cell infiltration. Generally, DPI increased the incidence and intensity of these changes in rats administered with doxorubicin. No signs of cardiomyocyte edema were found in the control and DOX groups. However, this histological phenomenon was found in the groups exposed to DPI alone and in combination with doxorubicin. The lower dose of DPI increased the incidence of cytoplasm vacuolization in rats receiving doxorubicin. Inflammatory cell infiltration and cardiac hypercontraction were most common in the DOX group and rare in the other groups. No signs of necrosis were observed in any animals. The absence of cardiomyocyte necrosis was confirmed in biochemical studies, since H-FABP and cTnI (Tab. 3) levels were not elevated. Interestingly, the level of cTnI was significantly lower in the DOX and DPI+DOX groups than it was in the control group. A similar change was found for cTnI in the 1DPI+DOX group. However, changes in H-FABP and cTnI levels observed in the DOX and DPI+DOX groups were insignificant. In all except the 0.25DPI+DOX group, the serum BNP level was lower than it was in the control, but no significant differences were found among them (Tab. 3). The cardiac glutathione level was at a similar level in all groups (Tab. 4). Doxorubicin had no significant effect on the cardiac NADPH level (Tab. 4), but DPI administered alone increased the cardiac NADPH level, compared with the level in the control group. However, when both compounds were administered together, the NADPH level was lower. Moreover, DPI significantly reduced the NADPH level in rats receiving doxorubicin, versus the DOX group, but the higher DPI dose normalized the MDA level elevated by DOX (Tab. 4). The cardiac NADH levels in the DPI + DOX groups (Tab. 4) were about three times as high as they were in the control, but there were no differences between the DPI + DOX and DOX groups.

Tab. 3. Serum level of cardiomyocyte disturbance markers (mean ± SD)

Group	H-FABP (ng/ml)	cTnI (ng/ml)	BNP (ng/ml)
Control	21.30 ± 4.46	0.96 ± 0.55	0.29 ± 0.06
DOX	5.90 ± 1.34*	0.46 ± 0.30	0.19 ± 0.08*
1 DPI	8.22 ± 3.44*	0.41 ± 0.20	0.14 ± 0.01*
0.25 DPI	22.20 ± 10.02	1.87 ± 0.87	0.14 ± 0.03*
DOX + 1 DPI	6.80 ± 5.36*	0.20 ± 0.08*	0.17 ± 0.08*
DOX + 0.25 DPI	12.84 ± 7.98*	0.93 ± 0.91	0.25 ± 0.09

Explanations: *p ≤ 0.05 vs control

Tab. 4. Cardiac level of oxidative stress and reduced force markers (mean ± SD)

Group	Glutathione (nmol/g tissue)	MDA (nmol/g tissue)	NADPH (nmol/g tissue)	NADH (nmol/g tissue)
Control	160.89 ± 16.60	29.82 ± 3.83	19.95 ± 5.29	50.65 ± 16.60
DOX	172.67 ± 25.85	83.58 ± 10.23*	24.53 ± 6.79	70.56 ± 23.45
1 DPI	173.33 ± 24.89	29.55 ± 2.40	34.65 ± 12.93*	174.56 ± 20.28*
0.25 DPI	187.56 ± 20.69	31.53 ± 6.07	38.95 ± 9.12*	144.93 ± 39.37*
DOX + 1 DPI	156.89 ± 14.81	29.07 ± 1.01‡	8.85 ± 3.51*‡	63.18 ± 32.37
DOX + 0.25 DPI	172.22 ± 16.35	34.94 ± 5.01	6.46 ± 2.77*‡	61.40 ± 19.06

Explanations: *p ≤ 0.05 vs control; ‡p ≤ 0.05 vs DOX

The main role in the late cardiotoxicity of doxorubicin is ascribed to the free radical mechanism. In the last three decades, numerous studies have improved our knowledge of the beneficial role of free radical scavengers and antioxidants in the prevention of doxorubicin-related cardiotoxicity leading to congestive heart failure. However, none of those agents have proved beneficial in clinical practice (3, 34). In the present study, an alternative approach was tested. Instead of scavengers of free radicals, the authors used a flavoenzyme inhibitor to prevent the generation of reactive oxygen species.

Doxorubicin-induced cardiotoxicity may depend on glycoprotein p, which reduces the intracellular level of xenobiotics. That effect was demonstrated in cultured rat and human cardiomyocytes

(H9C2) exposed to doxorubicin (24, 33, 56). However, the influence of DPI on glycoprotein p has not been evaluated in the available literature. On the other hand, since intracellular ROS can inhibit glycoprotein p, it is possible that DPI inhibits the protein function by inhibiting NADPH oxidase, which causes an increase in the cellular doxorubicin level in cardiomyocytes and secondary higher toxicity. According to the authors' best knowledge, it is not possible to state that cardiotoxicity is related to the DPI-regulated uptake and efflux of anthracyclines. That effect was not evaluated for the efflux of other toxic DOX metabolites. It is also interesting whether the concomitant administration of DOX and DPI alters the anthracycline pharmacokinetics in terms of uptake and efflux. This issue, however, has not been studied yet.

The data obtained indicate that DPI showed a beneficial effect on the anthracyline-induced cardiac oxidative stress. However, the simultaneous treatment with DPI and doxorubicin, versus doxorubicin alone, resulted in more pronounced morphological changes.

It appears that many factors may contribute to the aforementioned failure to transfer the beneficial effects of free radical scavengers and antioxidants from laboratory to clinical practice. First, the applied doses of these compounds were extremely high, impossible to use in clinical practice. Moreover, in most of these studies, doxorubicin was administered once, at very toxic doses. Finally, the interval between the end of drug exposure and autopsy was too short, and did not reflect the real conditions leading to a congestive heart failure. In the current study, doxorubicin was administered once a week for ten weeks, and histological and biochemical outcomes were analyzed three weeks after the last dose of the drug. With this protocol it was possible to exclude that the changes in oxidative stress levels were not caused by direct doxorubicin-related radical production, but resulted from changes induced by reactive oxygen species. This aspect is very important when explaining why congestive heart failure may appear years after the doxorubicin therapy is completed. According to the current hypotheses, oxidative damage caused directly by DOX leads to mitochondrial DNA (mtDNA) damage, which, in turn, is responsible for organelle dysfunction (4, 28). Any changes in mitochondrial electron transfer result in overproduction of reactive oxygen species, damaging mtDNA (29). This vicious circle of toxic events may be repeated many times (49), and its onset is not accompanied by overt clinical heart failure symptoms. Finally, mitochondrial insufficiency results in the development of cardiac contractility dysfunction involved in heart failure (34, 46). Moreover, according to the authors' studies, no lethal effect was observed for the doxorubicin dose applied (1.5 mg/kg). However, a similar regimen, even at a dose of 0.8 mg doxorubicin/kg, results in adverse cardiac effects on the histological, ultrastructural, and biochemical levels (27).

Information provided in Tab. 1 shows that numerous enzymes are involved in doxorubicin-related free radical generation. Moreover, these enzymes are sensitive to DPI. The inhibition of these enzymes may be expected to temper the univalent reduction of doxorubicin, resulting in the reduction of anion superoxide generation, ultimately decreasing oxidative stress. In the present study, a significant increase in the cardiac MDA level observed in rats treated with doxorubicin proves that this chemotherapeutic is responsible for oxidative stress. However, no changes were observed in the total glutathione level in the DOX group versus the control group. Oxidative stress is triggered by doxorubicin, but a few weeks after the drug is withdrawn, biochemical signs of oxidative stress are mainly restricted to mitochondria, and are not manifested so clearly in tissue homogenates (4). Sometimes, no changes in TBARS were observed even for a higher DOX dose (2 mg/kg) (40).

DPI injected alone has no impact on the cardiac MDA level. No similar data are available in the literature, but DPI increased the MDA level in N11 glial cells (39). The compound prevented cardiac lipid peroxidation caused by doxorubicin. NADPH is necessary to maintain the redox balance to avoid oxidative stress. This cofactor is critical for the regeneration of glutathione – the main redox cellular buffer. In the DPI + DOX group, no changes in the glutathione level were seen, but the NADPH level dropped significantly. It is very interesting because a significant increase in the NADPH level occurred when DPI was administered alone, whereas doxorubicin given alone did not cause significant changes in the NADPH level. The data indicate a close interaction between the two compounds. It seems probable that, paradoxically, NADPH is also used as a cofactor in a univalent enzymatic reduction of doxorubicin. In such a case, when both compounds are administered separately, protective mechanisms are sufficient to keep NADPH at a proper or higher level. However, when DPI and doxorubicin are administered together, the NADPH pool is extremely low. An interaction between the two compounds in relation to the cardiac NADH level was also observed. In the DPI groups, NADH was about three times as high as in the control and doxorubicin-exposed groups, in which changes were insignificant. On the other hand, changes in the NADH levels in the DPI + DOX groups were insignificant compared with those in the control group. It has been known for a long time that both compounds can inhibit the mitochondrial electron transport chain, but mechanisms underlying this phenomenon differ (6, 38). It is possible that DPI inhibits the respiratory chain, and, in consequence, NADH is insufficiently utilized. This may be the cause of the elevated NADH level. On the other hand, the doxorubicin-related respiratory chain inhibition relies on the use of NADH (being the complex I cofactor) for univalent reduction. When both compounds are administered simultaneously, the

two mechanisms may compensate each other, and the NADH level remains almost unchanged.

Cardiac oxidative stress initiated by free radicals has many implications, ultimately leading to a cardiac hypertrophic remodeling and cell death (47, 49, 54), but in the current study no signs of remodeling were found in any of the groups. Specific blood biochemical markers for cardiomyocyte necrosis (HFABP and cTnI) (8, 23, 37) revealed no signs of this phenomenon. Additionally, no increase in the BNP level – a cardiac function marker (15) – may indicate the lack of disturbances in cardiac muscle stretching.

Besides changes in morphology and MDA elevation, the cardiac toxic effect in the DOX group was visible in decreased FABP, cTnI, and BNP levels, compared with those in the control. Clinically, an increase in serum levels of these parameters is only of diagnostic importance. However, a significant decrease in these markers should also be discussed from the scientific point of view. A cellular response to xenobiotics, which is usually manifested as apoptosis, necrosis, and atrophy, varies depending on the administration regime and the study duration, and especially on the interval from the last doxorubicin injection. The authors postulate that such a clear reduction in these proteins may be secondary to atrophy, resulting in the inhibition of synthesis and intensified lysosome-dependent protein catabolism. Singal et al. (43) revealed that in animals treated with doxorubicin for 6 weeks the number of heart lysosomes per unit area increased 10 times from the control level. Other authors indicate that BNP and cTnI gene expression in subjects treated with the drug is significantly reduced (2, 7, 41). Additionally, this atrophy thesis is consistent with our morphological observation, presented in Tab. 2, and particularly with vacuolization resulting from lysosome fusion with endoplasmic reticulum. Moreover, oxidative stress may be a factor causing fibrosis observed in the doxorubicin-treated group (44). Histopathological examination confirmed the absence of cardiomyocyte necrosis in all groups. DPI induced fibrosis, edema, and cell infiltration. These structural changes may be deleterious to the heart function and thus indicate a harmful potential of DPI. However, DPI increased the incidence rate and intensity of interstitial and perivascular fibrosis, as well as interstitial edema, in rats administered with doxorubicin. Unlike in the DPI and DPI+DOX groups, no cardiomyocyte edema was observed in the control and DOX groups. DPI increased the incidence of cytoplasmic vacuolization in rats receiving doxorubicin. However, the main limitation of the study was a relatively high incidence of similar histological changes in groups exposed exclusively to DPI. Although they were observed locally, such abnormalities have not been previously found. On the other hand, the data obtained may influence clinical application of DPI.

Concluding, the present study confirms that oxidative stress and cardiac morphological changes are present even three weeks after completing doxorubicin administration in rats. DPI protects against oxidative stress caused by doxorubicin. NADPH probably plays a crucial role in this phenomenon, as cardiac NADPH levels in the DPI + DOX groups were extremely low. Generally, DPI augments cardiac morphological changes caused by doxorubicin. Because DPI protects against doxorubicin-related oxidative stress and, at the same time, leads to increased morphological changes, it may be concluded that oxidative stress does not directly cause cardiac histopathological changes. In future studies in a similar model, longer intervals after the end of doxorubicin administration and lower DPI doses should be tested. The data obtained cannot be transferred directly to human clinical practice because of the number of metabolic differences between humans and rats. Other studies in non-rodent models, particularly in animals with previously induced neoplasms, are also desirable.

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