Effects of Dexrazoxane and Amifostine on Evolution of Doxorubicin Cardiomyopathy *In Vivo*

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Doxorubicin is one of the most active drugs in oncology, with cardiotoxicity as a serious side effect of its application. The aim of this study was to investigate dexrazoxane and amifostine impact on the evolution of myocardial changes induced by doxorubicin. BalbC female mice were treated with doxorubicin only (10 mg/kg, single intravenous push), or with dexrazoxane (200 mg/kg, intraperitoneal [ip]) or amifostine (200 mg/kg, ip) 60 mins or 30 mins prior to treatment with doxorubicin, respectively. Blood sampling for determination of conventional serum-marker activity was performed 48 hrs later. The grade of histopathology changes was evaluated by light microscopy 1.5 and 3 months after treatments using the Billingham scoring method. Control groups consisted of nontreated mice. After doxorubicin-only treatment, the grade of heart tissue damage was found to increase in the period between 1.5 and 3 months. A similar but less intense progression was also detected in amifostine-pretreated animals, with significant difference among median Billingham scores between the two time points. The pretreatment with dexrazoxane suspended expansion of tissue lesions in time. Changes in serum enzyme activity revealed two correlations: the greater reduction in alpha-hydroxybutyrate dehydrogenase (α-HBDH) leakage is associated with a lower percentage of damaged tissue, and the creatine kinase to α-HBDH percent of difference ratio being greater than one is correlated with limited spreading of pathological lesions. Our results indicate that the development of doxorubicin-induced heart failure is based on a slow and persistent expansion of pathological process even long after the completion of the treatment. Dexrazoxane has proved to be successful and superior over amifostine against such an evolution of doxorubicin cardiomyopathy. Exp Biol Med 232:1414–1424, 2007

Key words: evolution of doxorubicin cardiomyopathy; dexrazoxane; amifostine; *in vivo*

Introduction

Antineoplastic antibiotics of the anthracycline class are widely used, but they are also very well known for their cardiotoxicity. The probability of cardiotoxicity is clearly related to cumulative anthracycline doses and the rate of each dose application. Studies have reported that 50% of patients administered cumulative doses above those recommended (450 mg/m^2 for doxorubicin and 1000 mg/m^2 for epirubicin) would experience measurable functional impairment, and up to 26% of such patients would develop congestive heart failure (CHF; Ref. 1). Long-term follow-up studies suggest that cardiotoxicity occurs in as many as 71% of patients treated with anthracyclines in childhood (2). CHF, generally refractory to common medications, is mainly regarded as a consequence of chronic doxorubicin administration, but its development was also confirmed in patients who received just a single dose. It usually develops within a year after the completion of anthracycline-based therapy, but very late forms of cardiac dysfunction have been also described (2).

A few approaches have been tested in an attempt to lower the risk of cardiac damage. Because cardiotoxic activity of doxorubicin appears to correlate with the first peak plasma level, but not with the area under the curve (3), its administration as a continuous infusion that lasts over 96 hrs or as a liposomal formulation has had some success (4, 5). The former method is not widely accepted because it requires a central venous catheter or a pump, which is inconvenient for the patient, and carries the risk of infection. Liposomal delivery of anthracyclines may provide lower cardiotoxicity,

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but may increase the incidence of other toxicities, such as palmar-plantar erythrodysesthesia and neutropenia (6). Liposomes have not yet found a place in routine practice due to both the need for more long-term data regarding their efficacy and toxicity and the high cost of these products. Therefore, the use of a cardioprotective agent during anthracycline therapy is imposed as the best strategy so far.

Among the many investigated cardioprotective agents, dexrazoxane (ICRF-187) was found to be the most promising. Its efficacy against doxorubicin cardiotoxic activity was confirmed in a series of prospective clinical trials (7–9). However, the possibility of its interference with the tumor response rate to the anthracycline treatment (8, 10) and higher incidence of myelodysplastic syndrome (11–13) increase the anxiety of clinicians to use dexrazoxane. Moreover, dexrazoxane is not proven to provide protection against late cardiovascular effects of doxorubicin (14, 15), which additionally reduces motivation for its use.

In most preclinical studies, the cardioprotective activity of various agents was tested either against higher doses of doxorubicin in order to evaluate acute effects within days (16, 17), or against repeated doxorubicin administration in lower doses with the evaluation done months after treatment initiation but no later than a month after its termination (18–20). In our previous study, we used a unique experimental model in which doxorubicin was applied as a single bolus at its maximal tolerated dose (MTD), and the assessment of histological changes occurred long after doxorubicin had been systemically cleared (21). Such a model was utilized preferentially to follow the effect of d,l-alpha-tocopherol (vitamin E) on the development of doxorubicin-induced cardiomyopathy. Indeed, it afforded a relevant profile of vitamin E cardioprotective activity, but also provided a new viewpoint on the evolution of doxorubicin cardiomyopathy (21).

In accordance with our previous investigation, the same experimental model was applied in the current study, the primary aim being to explore the effect of dexrazoxane on late manifestations of doxorubicin cardiotoxicity. Alongside dexrazoxane we tested amifostine, which has already been examined for its cardioprotective activity (19, 20, 22–24), but the published results also lack descriptions of its impact on delayed myocardial changes. The ultimate assessment endpoint in current research was the light microscopy evaluation of the heart muscle specimens performed 1.5 and 3 months after the treatments. Additionally, the activities of conventional serum enzymes were measured 48 hrs after the treatments. Knowing that aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and creatine kinase (CK) are not particular indicators of cardiotoxicity, we deliberately chose them along with alpha-hydroxybutyrate dehydrogenase (α-HBDH), which is a more specific marker of heart muscle deterioration and the least investigated in cases of doxorubicin toxicity (25). The idea was not to reinvestigate their significance as individual predictors, but to observe correlations between changes in their activity and to detect a possible pattern that could be recognized as a predictive marker for dexrazoxane and amifostine influence on the evolution of doxorubicin-induced cardiomyopathy.

Materials and Methods

Animals. BalbC/NIH female mice (Military Academy of Medicine, Belgrade, Serbia), 9 weeks old, were housed in cages (up to 15 per cage) under constant temperature and humidity conditions, 12:12-hr light:dark cycle, with food and water available *ad libitum*. Blood sampling for biochemical analysis was performed by orbital sinus puncture while animals were under a light diethyl ether anesthesia; afterward, they were euthanized with an overdose of sodium pentobarbital. An overdose of sodium pentobarbital was also used when animals were sacrificed in order to remove hearts, which were later analyzed for histopathology changes. All procedures involving animals were approved by the Ethical Committee of the National Cancer Research Center of Serbia, in compliance with the Guidelines for the Use and Care of Laboratory Animals (NIH Publication 85–23).

Drugs and Chemicals. Doxorubicin (10 mg; Adriamycin; Pharmacia-Upjohn Company, Kalamazoo, MI) and amifostine (500 mg; Ethyol; Schering-Plough Pharmaceuticals, Kenilworth, NJ) were diluted with 4 and 10 ml saline, respectively. Further dilutions were made with the same solvent to the final concentrations in correspondence to doses. Dexrazoxane (500 mg; Cardioxane; Chiron Corporation, Amsterdam, Netherlands) was initially diluted with 1.5 ml normal saline, followed with 3.5 ml of Ringer lactate (Hemofarm, Vrsac, Serbia). Further dilutions were made with Ringer lactate only. By this dissolving procedure, the pH of final dexrazoxane solution was 5.8. All drugs were prepared immediately before usage, with sterile solvents and under sterile conditions. The controls and reagents for detection of AST, LDH, α-HBDH, and CK serum activity were purchased from Dialab GmbH, Vienna, Austria.

Experimental Protocol. Animals were randomly divided into three groups: Group I, animals treated with doxorubicin only (10 mg/kg, 25 mice); Group II, animals treated with amifostine (200 mg/kg, 24 mice) 30 mins prior to doxorubicin; Group III, animals treated with dexrazoxane (200 mg/kg, 20 mice) 1 hr prior to doxorubicin. The full dose of doxorubicin was applied in a single injection (0.1 ml, intravenous [iv] push) to each experimental group. Amifostine and dexrazoxane were also administered once in full doses as 0.1-ml and 0.2-ml intraperitoneal (ip) injections, respectively. The sera for biochemical analysis were taken 48 hrs after drug application, whereas hearts for histopathology examination were obtained 1.5 and 3 months after drug treatment, as established previously (21). The nontreated control group altogether consisted of 40 animals (30 sacrificed after blood sampling and 5 at 1.5 and 3 months, respectively, for histopathology examination).

Biochemical Analysis. Biochemical tests of sera included activity of AST, LDH with its isoenzyme α-HBDH, and CK. The blood specimens were collected in safe-lock centrifuge tubes (2 ml) and left for 1 hr at room temperature to allow spontaneous coagulation. The segregated sera were carefully compiled, stored as 50-μl aliquots at –20°C, and assayed within 72 hrs. All aliquots with noticeable hemolysis were removed from further process immediately. Serum parameters were analyzed using a multi-channel analyzer (Abbott Spectrum CCX, Abbott Laboratories, Abbott Park, IL). Calibration of the equipment was against diluent controls for each of the parameters assayed. All assays were performed according to the manufacturer's instructions. The reaction to α-HBDH activity was maintained under conditions that are more sensitive to LDH 1 and LDH 2 isoenzymes than to LDH 3, 4, and 5 isoenzymes (phosphate buffer [pH 7.2], 30°C, and alpha-oxobutyrate as a substrate instead of pyruvate). Activity measurements were expressed in international units of enzyme activity per liter (IU/liter) of serum. The percent of α-HBDH (%α-HBDH) was estimated by using the formula: %α-HBDH = (α-HBDH/total LDH) × 100. The reference change limits were obtained from sera of 30 nontreated mice (control group).

Histopathology Examination. After the heart was removed, the right and left ventricles were transected into sections parallel to the atrioventricular sulcus according to the method of heart preparation and placed in 10% buffered formalin for 24 hrs. Further fixation of the heart tissues and paraffin embedment was carried out according to standard procedures. The obtained specimens were stained with hematoxylin-eosin, respecting conventional protocols. The extent of myocardial damage was graded according to Billingham scoring rules (26) as follows: Grade 0, cells show no anthracycline damage; Grade 0.5, the myocardium is not completely normal, but no anthracycline-specific changes are evident, Grade 1.0, few cells (< 5%) have myofibrillar loss or distended sarcoplasmic reticulum, or both; Grade 1.5, small groups of cells (5%–15%) show anthracycline effects consisting of marked myofibrillar loss or cytoplasmic vacuolization, or both; Grade 2.0, 16%–25% of cells demonstrate the described changes; Grade 2.5, 26%–35% of cells demonstrate the described changes; Grade 3.0, specimens exhibit diffuse cell damage, with more than 35% of cells showing pathologic changes, loss of contractile elements and organelles, and mitochondrial and nuclear degeneration. The heart tissue specimens were analyzed by three pathologists as a blind investigation. The results were recorded as the median of three independent scores for each animal (individual score). Ten animals per group were included; five were sacrificed at 1.5 months and the rest at 3 months after treatment. Animals from the nontreated control group were kept and sacrificed 1.5 and 3 months from the beginning of the experiment.

Data Analysis. The results of the serum enzyme activity were analyzed using the conventional statistical method and the percent of difference (%difference). The Kruskal-Wallis test with a Mann-Whitney posttest were performed in order to evaluate the statistical significance of the activity of each enzyme separately, between all experimental groups (including nontreated control group). The %difference was calculated for enzyme activity using the mean values, as follows:

$$\%\ \text{difference} = \frac{\text{mean D} - \text{mean P}}{\text{mean N} - \text{mean D}} \times 100,$$

where D is the enzyme activity in the group treated with doxorubicin only, P is the enzyme activity in the group treated with a combination of an investigated protector (amifostine or dexrazoxane) and doxorubicin, and N is the enzyme activity in the nontreated control group. The statistical significance of the histopathology results (expressed as median scores according to Billingham scoring rules) was analyzed by comparing individual animal scores between all experimental groups (including nontreated control) for each time point of evaluation separately (1.5 and 3 months) with the Kruskal-Wallis test and a Mann-Whitney posttest. In order to estimate significance in the progression of pathological damage over time, a Mann-Whitney test was used to compare individual scores between 1.5 and 3 months within the same treated group.

Results

Previously, we established the optimal time points for blood (48 hrs) and heart tissue sampling (1.5 and 3 months; Ref. 21). Supporting reduction of the number of sacrificed animals in control groups, we conducted a large, blood-sampling-only pilot experiment on a single nontreated control group, groups treated with cardiotoxic drugs, and multiple experimental groups treated with various cardioprotective tested agents. Therefore, values for enzyme activities in the nontreated control and the doxorubicin-only–treated group presented here are identical to those previously published (21). Apart from that, histopathology examinations of the nontreated control group, the doxorubicin-only–treated group, and the pretreated experimental groups were organized separately.

The doses of investigated protectors were chosen according to literature data. For dexrazoxane, the most efficient dosing ratio to doxorubicin in animal experiments was 20:1 (27), but because of severe leucopenia as a dose-limiting toxicity, its dose is restricted to 10:1 in man (12). In contrast to dexrazoxane, the dose of amifostine has never been discussed in relation to doxorubicin, and amifostine was applied in a range from 2.3 (20) to 200 times that of the doxorubicin dose (28). Also, the activity of both agents on animal models was investigated mostly against repeated administration of a low dose of doxorubicin (18–20, 28, 29). Because in the current study the cardiotoxic agent was given in a booster shot, we decided to apply both tested drugs in a 20:1 dose ratio to doxorubicin.

It was necessary to find the best procedure for

Table 1. Serum Enzyme Activities of AST, LDH, α-HBDH, and CK in Mice Under Different Treatments[a]

Treatment (mg/kg)	No. of mice	AST (IU/liter)	LDH (IU/liter)	α-HBDH (%)	CK (IU/liter)
Nontreated	30	19.93 (1.39)	121.80 (9.89)	13.86 (1.00)	27.00 (5.58)
Doxorubicin (10)	15	33.00 (4.05)[b**]	205.60 (12.99)[b**]	24.66 (1.51)[b**]	51.73 (5.81)[b**]
Amifostine (200) + doxorubicin (10)	14	27.07 (3.27)[b**c**]	184.29 (32.51)[b**]	19.84 (2.90)[b**c**]	43.93 (8.98)[b**c*]
Dexrazoxane (200) + doxorubicin (10)	10	19.20 (2.90)[c**d**]	123.50 (12.91)[c**d**]	17.69 (1.55)[b**c*d**]	28.30 (4.55)[c**d**]

[a] Enzyme activity was analyzed in mice sera prepared 48 hrs after drug application. All drugs were applied in a single bolus injection. In combined treatments, amifostine was given 30 mins before doxorubicin, and dexrazoxane was given 1 hr before doxorubicin. Data represent mean (standard deviation). For statistical evaluation, the Kruskal-Wallis test and the Mann-Whitney posttest were used.
[b] Significantly different compared to nontreated control group.
[c] Significantly different compared to doxorubicin-only–treated group.
[d] Significantly different compared to amifostine + doxorubicin–treated group.
* $P < 0.05$; ** $P < 0.01$.

preparation of dexrazoxane solution and a route for its application that differs from those previously used (18, 28, 29). When dexrazoxane is dissolved either in *aqua pro injectione* or normal saline, the pH is approximately 1.6. Our attempts to deliver this solution by iv push in one of four existing tail veins resulted in its vigorous postinjection dilatation, together with extreme constriction of the other three veins. Any effort to introduce doxorubicin into the same blood vessel up to 2 hrs afterwards failed due to vain rupture followed by doxorubicin extravasation. In previous reports, such solution was applied by ip route in studies with repeated dosing schedules designed to follow body weight changes, survival, and neutrophil count (18, 28, 29). Significant changes of these parameters were not recorded in any of these reports when comparing treated and nontreated animals.

To avoid the development of a serious irritation or even necrotic lesions in the peritoneal cavity, we decided to prepare dexrazoxane solution with Ringer lactate after its initial dissolution with minimal necessary volume of normal saline (1.5 ml). After this manipulation the pH of dexrazoxane solution was increased to approximately 5.8. In the additional pilot experiment, this solution was applied to mice by ip route, but did not induce changes in serum enzymes measured at 48 hrs, and no signs of previous inflammation in abdominal cavity were found at necropsy performed 1.5 and 3 months later (data not shown).

The changes in serum enzyme activities in doxorubicin-only–treated mice compared to nontreated control revealed intensive destruction of myocardial tissue (Table 1), with the pattern quite similar to that in myocardial infarction (21). However, the true severity of the heart muscle injury became evident after examination by light microscope. At the first evaluation, performed 1.5 months after doxorubicin-only treatment, the left ventricle wall was thinner, with dilated cavity, compared to control specimens (Figs. 1 and 2A and B). Cells with morphologic changes typical for doxorubicin toxic activity were organized in plaques allocated in the subendocardial area of the muscle. Along the plaque edges, a layer of cells with initial vacuolization of cytoplasm and interstitial edema formed a demarcation zone to unchanged heart tissue. Plaques were preferentially distributed in the trabeculae, apex, septum, and especially anterolateral wall, where plaque was thicker in comparison to other regions (Fig. 2C and D). In the posterior wall, plaque with a demarcation zone spread along a narrow space in the vicinity of left ventricle cavity (Fig. 3), whereas only a few altered cells could be found in the right ventricle wall. It is obvious that the parts of the heart muscle with the highest oxygen consumption are the most vulnerable to doxorubicin toxicity. In most of the tissue samples, about 20% of the heart muscle was overtaken with pathologic changes, with a median score of 2.0 according to Billingham scoring rules (Table 2). Thinning of the anterolateral wall and consequential dilatation of the left chamber, together with compensative hypertrophy of the posterior wall, increased with time (Fig. 4A and B). These changes indicate progression toward dilative cardiomyopathy, which is known as the final sequel of doxorubicin toxic activity against heart tissue. Nevertheless, an additional type of progression was recognized in specimens of animals sacrificed 3 months after the treatment. The plaque area and demarcation zone were extended deeper into the muscle, though the expansion process was not equally aggressive all over the heart. The anterolateral wall was mostly affected, almost completely abounded with myocytes in the advanced stage of degeneration and the loss of a demarcation zone (Fig. 4C and D). The same degree of change was found in frontal part of the heart septum. Expressed in numbers, one third of the heart tissue specimens were pathologically altered, with a median group score of 2.5 (Table 2).

It is important to make a distinction between the grade of myocardial pathological damage estimated by Billingham scoring method and the grade of the heart functional impairment. The Billingham score represents percentage of harmed tissue, regardless its distribution over the heart muscle. The 30% of damaged cells mostly allocated to a relatively restricted area, such as the anterolateral wall, is assumed to result in heart dysfunction more severe than if

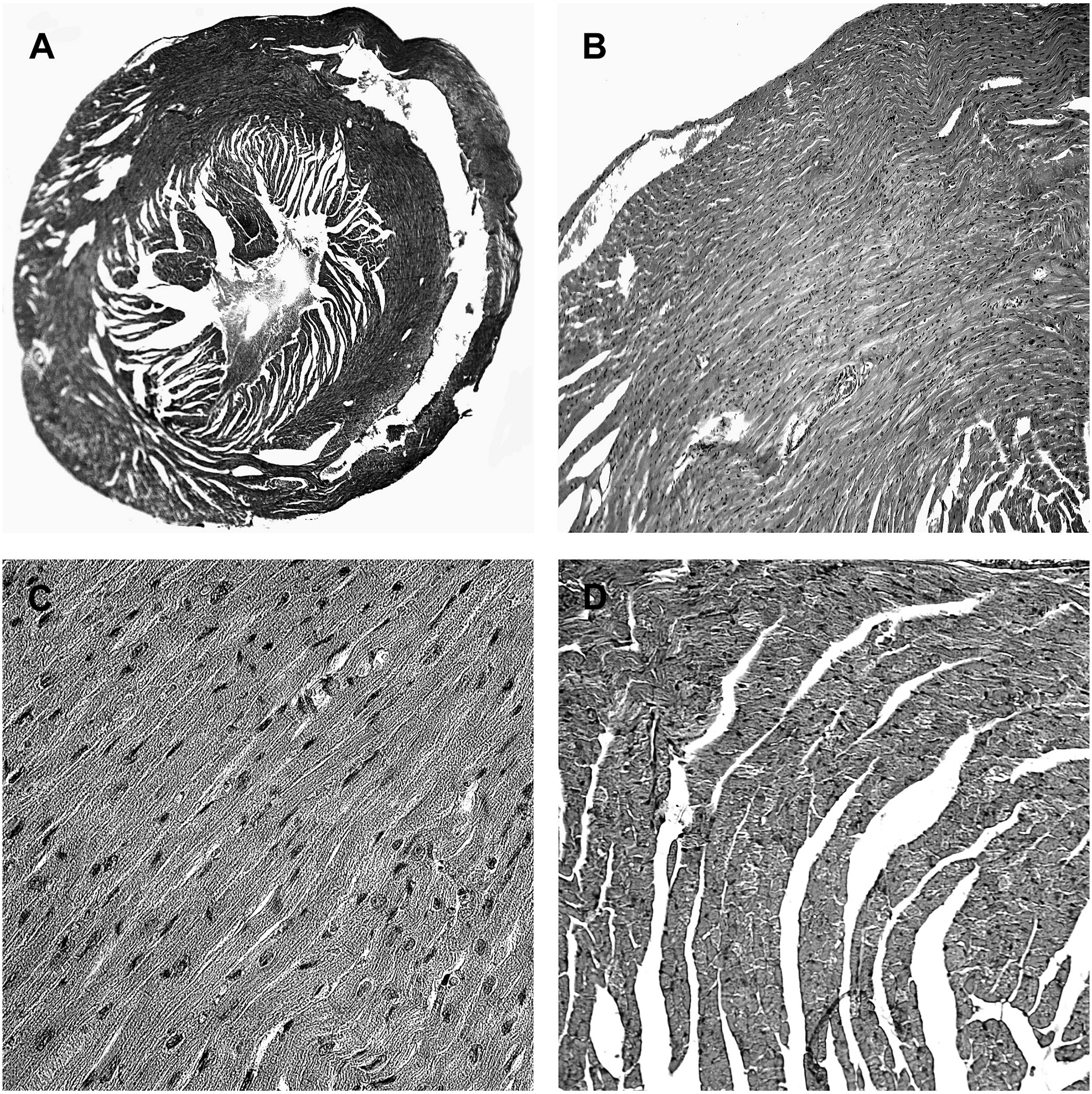

Figure 1. The morphology of the heart from nontreated mouse: gross section (A), light microscopic details of the anterolateral wall (B and C, magnification: ×100 and ×400, respectively), and trabeculae (D, magnification: ×200).

they were distributed equally through the myocardium. Therefore, it was expected that the investigated protectors would reduce the size of the doxorubicin-induced primary lesion, and particularly to limit further expansion of the pathological process within the most vulnerable parts of the heart muscle.

The protective activity of dexrazoxane and amifostine against doxorubicin cardiotoxicity was more than obvious as early as 1.5 months after treatment. In the heart specimens of both experimental groups, altered myocardial cells exhibited all the typical characteristics of anthracycline cardiac toxicity, but the only difference compared to the doxorubicin-only–treated group was in the quantity of affected myocardium. With the addition of amifostine, the number of pathologically changed cells was in the wide range from less than 5% up to one quarter of the whole organ (Table 2). In dexrazoxane treated mice, it did not exceed more than 15%; indeed, in four of ten animals in the dexrazoxane group, the anthracycline-specific changes were not evident (scored as 0.5; Table 2). Those heart specimens

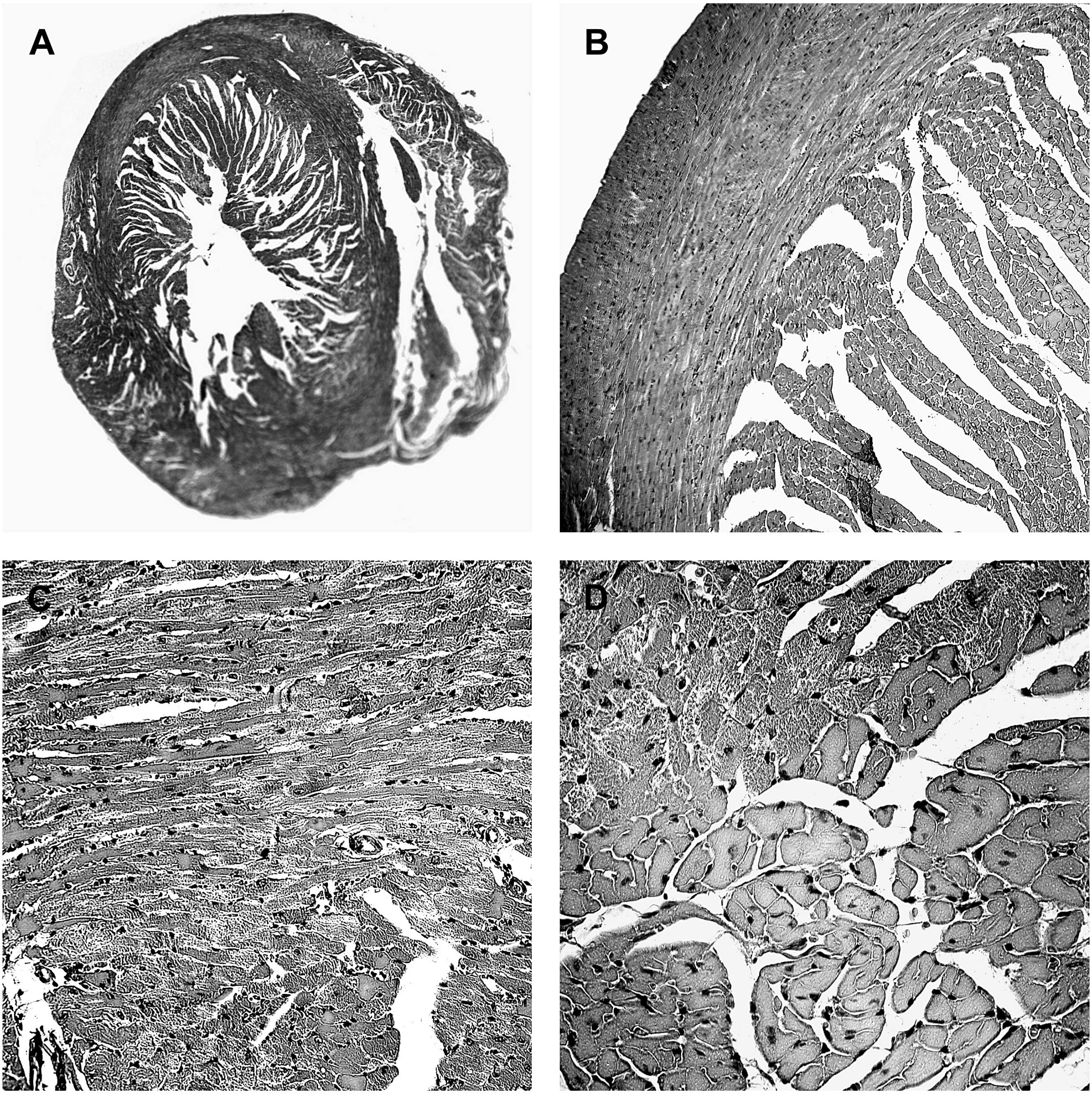

Figure 2. The heart morphology of a mouse 1.5 months after doxorubicin-only treatment. Transversal gross section shows changed heart shape, elongated trabeculae, thinned septum and anterolateral wall, and thicken right ventricle wall compared to nontreated control specimen (A). Light microscopy of anterolateral wall (B, magnification: ×100), with the plaque in the subendocardial area consisting of myocytes in different stages of degeneration, from cells with small cytoplasmic vacuoles and condensed nuclei to "ghost cells" enlarged in size with homogenized cytoplasm and paled nuclei (C, magnification: ×200) and trabeculae entirely overtaken with "ghost cells" (D, magnification: ×400).

were described by pathologists as exhausted myocardium due to moderate oxygen deprivation or by risen afterload, with sporadic myocyte attenuation and pleomorphism. Such changes can be initial signs of myocardial hypertrophy, but in the 5 animals sacrificed at the end of the experiment, hypertrophy was not verified. The difference in the impact of dexrazoxane and amifostine on the evolution of doxorubicin cardiomyopathy became fully obvious after the examination performed at 3 months (Table 2). In dexrazoxane-treated mice, only three out of five had doxorubicin-specific altered cells, which overtook less than 5% of the heart muscle. On the other side, significant progression in the course of dilative cardiomyopathy was evolved in the amifostine group. The histological changes were almost the same as after doxorubicin-only treatment, with the exception of the demarcation zone, which was

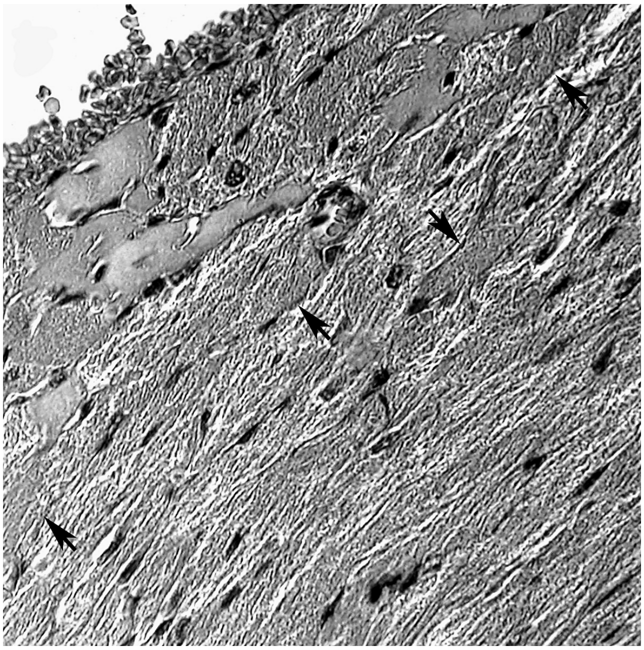

Figure 3. The light microscopy of the posterior ventricle wall from an animal treated with doxorubicin only and sacrificed 1.5 months later (magnification: ×500). Typical plaque formation: thin layer of "ghost cells" distributed along subendocardial edge, followed by demarcation zone consistinh of cells with initial vacuolization of cytoplasm, myofibril disintegration, and condensed nuclei (cells indicated with arrows), surrounded with interstitial edema to healthy tissue.

narrow with better defined borders to a wider area of unaffected tissue in the anterolateral left ventricle wall. Also, the statistical test confirmed significant progression in amifostine-treated mice between 1.5 and 3 months, with loss of significance compared to the doxorubicin-only–treated group (Table 2).

The results of serum analysis afforded additional information that was useful for a more accurate assessment of the short- and long-term effects of dexrazoxane and amifostine after doxorubicin administration. As presented in Table 1, in the sera of animals treated with amifostine, LDH was the only enzyme not significantly reduced compared to the doxorubicin-only–treated group and was logically least reduced when expressed over %difference (Fig. 5). Amifostine was proclaimed as a pluripotent protector (30), but persistent LDH activity suggests that amifostine is unable to provide adequate protection against doxorubicin toxicity in various tissues. However, dexrazoxane, which works as a protective chelator (31) and as a topoisomerase II inhibitor (i.e., a weak cytotoxic drug; Refs. 32, 33) achieved almost complete reduction of LDH leakage induced by doxorubicin. Dexrazoxane's ability to protect the kidneys in addition to the heart muscle was previously reported (18, 29), but it is hard to believe that the level of doxorubicin used in the dosing schedule in this study would affect only these two organs. Therefore, the crucial mechanism responsible for doxorubicin early toxic events seems to be its interaction with ferric ions, and dexrazoxane appears to be a far more potent protector on a whole-body level than was appreciated.

We previously discussed the reliability of change in α-HBDH activity as a marker for the grade of myocardial damage, and discarded the option that it could also be observed as a marker for doxorubicin nephrotoxic activity (21). It was the only serum marker that stayed significantly elevated after addition of dexrazoxane compared to the nontreated group (Table 1), which indicates incomplete protection of the heart muscle. Indeed, in the heart specimens of all animals treated with dexrazoxane, histo-

Table 2. Billingham Scores of Heart Tissue Specimens Estimated 1.5 Months and 3 Months After Treatment[a]

	Billingham scoring grade							
Treatment (mg/kg)	0	0.5	1	1.5	2.0	2.5	3	Median score
1.5 months								
Nontreated control	4	1						0
Doxorubicin (10)					4	1		2.0^{b**}
Amifostine (200) + doxorubicin (10)			2	2	1			1.5^{b**c*}
Dexrazoxane (200) + doxorubicin (10)		2	2	1				1.0^{b*c**}
3 months								
Nontreated control	3	2						0
Doxorubicin (10)					1	4		2.5^{b**}
Amifostine (200) + doxorubicin (10)					3	2		2.0^{b**e*}
Dexrazoxane (200) + doxorubicin (10)		2	3					$1.0^{b*c**d**}$

[a] The heart tissue specimen of each animal was analyzed and graded by three independent pathologists according to Billingham scoring rules, and the final result was recorded as the median of three scores (individual score). Ten animals per group were included; five were sacrificed at 1.5 months and the rest 3 months after treatment. Table results are expressed as the individual score for each animal and median score per group. Statistical evaluation between groups at the same time of estimation was performed using the Kruskal-Wallis test and the Mann-Whitney posttest. Separately, the Mann-Whitney test was used to calculate significance within the same treated group between 1.5 and 3 months.

[b] Significantly different compared to nontreated control group.

[c] Significantly different compared to doxorubicin-only–treated group.

[d] Significantly different compared to amifostine + doxorubicin treated group.

[e] Significantly different compared to animals under the same treatment sacrificed 1.5 months after treatment.

* $P < 0.05$; ** $P < 0.01$.

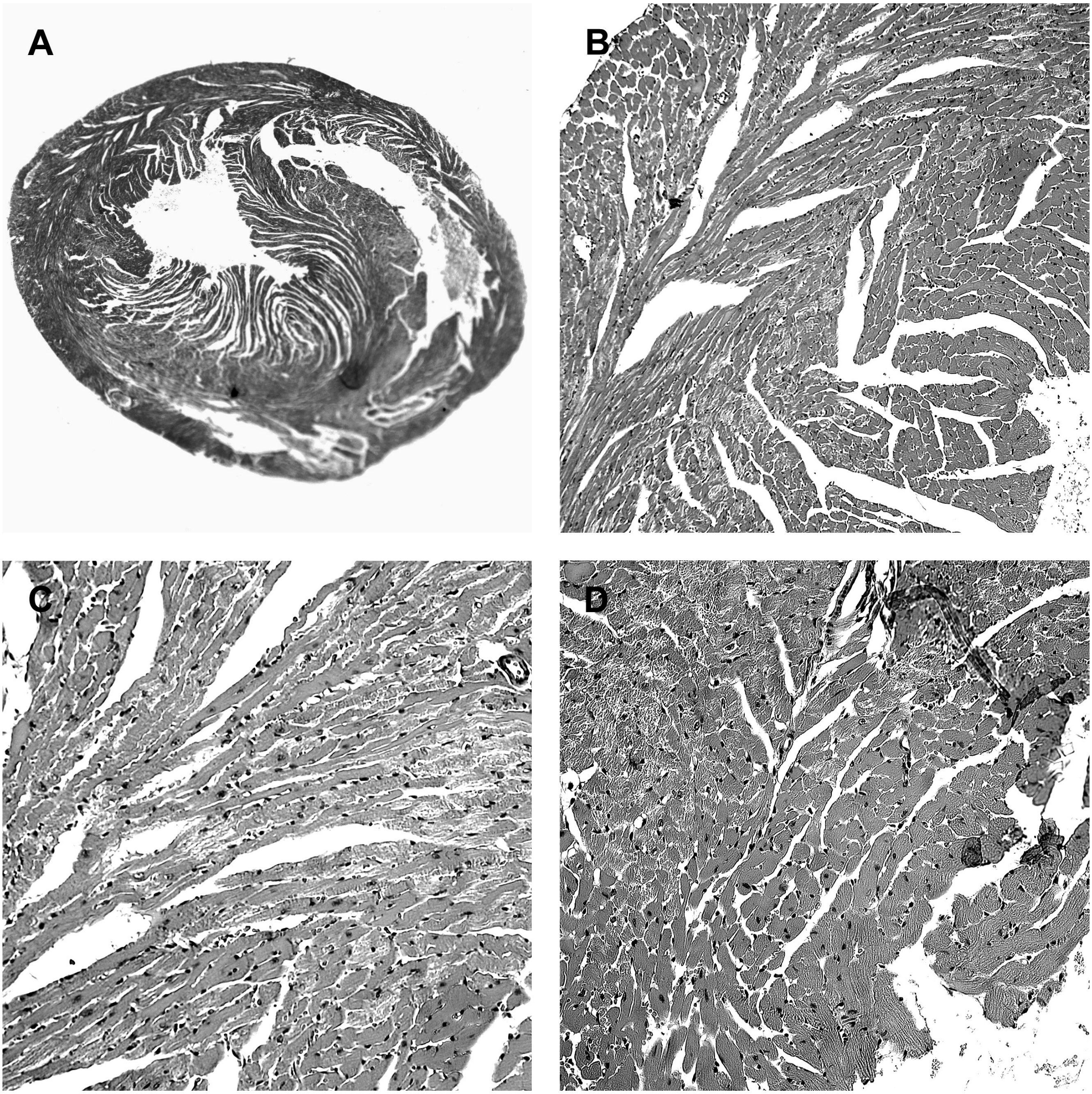

Figure 4. The heart morphology of a mouse 3 months after doxorubicin-only treatment. Transversal gross section indicates changes, including dilated cardiomyopathy, trabeculae of the anterolateral wall and frontal part of the septum are drastically shortened, and septum prolapsing into the left chamber. The right cavity is extended and rounded due to significant hypertrophy of the posterior wall (A). Light microscopic details of severely damaged anterolateral wall (B, magnification: ×100), with almost all cells in advanced stage of degeneration and disappearance of demarcation zone (C, D, magnification: ×200).

logical changes were found, some specific for doxorubicin cardiomyopathy, others nonspecific. However, dexrazoxane achieved greater reduction of α-HBDH leakage (Fig. 5) and higher levels of myocardial protection when compared to amifostine (Table 2). Except for the apparent relation between %difference of α-HBDH activity and the extent of pathological changes in the heart muscle, there were no obvious correlations between changes in the activity of this and the other three enzymes that would allow more information about the impact of the investigated protectors on the size of the primary myocardial lesion induced by doxorubicin. However, the link between the ratio of CK and α-HBDH %difference greater than 1 and the effect of protectors on the evolution of doxorubicin cardiomyopathy was noticed. In the dexrazoxane- (Fig. 5) and the vitamin E–treated (21) groups, CK activity was reduced to a greater

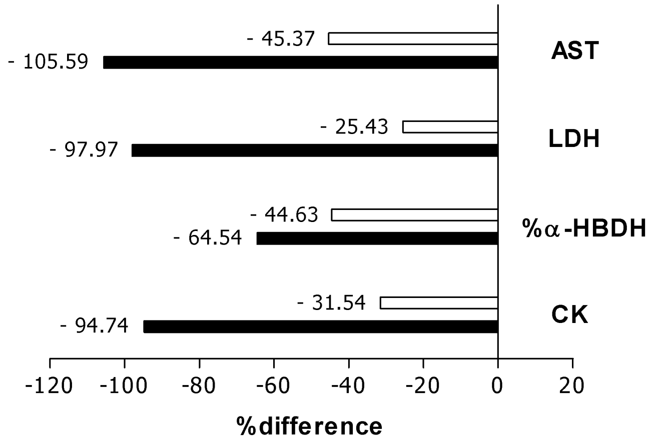

Figure 5. The %difference in AST, LDH, α-HBDH, and CK activity between the group treated with doxorubicin 10 mg/kg only and groups under combined treatments, amifostine 200 mg/kg and doxorubicin 10 mg/kg (opened bars), dexrazoxane 200 mg/kg and doxorubicin 10 mg/kg (closed bars). Presented values were calculated using the mean data of enzyme activity in sera of treated mice 48 hrs after drug application.

extent compared to α-HBDH, which correlated with their ability to reverse further extension of the primary lesion.

Discussion

Previous preclinical studies were designed starting with the observation that myocardial damage by doxorubicin is immediate and that each doxorubicin administration generates a loss of a certain quantity of muscle fibers, but physiological compensation prevents the occurrence of heart failure for a long time. Investigations *in vitro* are aimed at molecular understanding of doxorubicin cardiotoxicity and cannot help to elucidate the evolutionary aspects of CHF development. Most *in vivo* studies were organized to mimic the clinical conditions of the anthracycline therapy, with repeated doxorubicin administration in lower doses and evaluation performed during the treatment or a short time after its completion. Previously, we applied an original experimental design with the purpose other than tracking of time-dependent changes in histological grade of doxorubicin cardiotoxicity (21). The progression of changes between two time points of histological assessment in the doxorubicin-only–treated group was expected exclusively in the course of dilative cardiomyopathy. Moreover, analysis of the heart muscle specimens revealed an increase in the number of damaged heart cells, which is surprising because doxorubicin was given only once. The histopathology investigation in the current study was conducted as an independent experiment, but results for the doxorubicin-only–treated group are similar to those formerly published (21). Here we are not going to speculate about the underlying molecular mechanisms. The most important issue is that both present and previous data point to a progression in the grade of myocardial changes in time. Such findings are a sign of sustained spreading of biochemical reactions as the cause of myocardial destruction, initiated with a single doxorubicin application and continuing long after doxorubicin was systemically cleared. Thus, the apoptotic death of initially affected myocardial cells (34–36) and the simultaneous expansion of a specific pathological process resulting in the loss of more functional heart muscle units are the basic mechanisms in the development of delayed dilative cardiomyopathy induced by doxorubicin. Therefore, the true efficacy of some cardioprotective agent should not be estimated only according to its ability to reduce the size of the primary lesion, but also its ability to terminate further spreading.

Under the term “primary lesion” we designate a part of the heart muscle which was affected straight away with doxorubicin administration. Its exact size could be estimated only with the ultramicroscopic examination within a few days after the treatment. Pathologically changed portions of the heart muscle seen under the light microscope after 1.5 months did not necessarily correspond to the size of the primary lesion, and most probably do not. As the present study elucidates, the evolution of doxorubicin cardiomyopathy is a dynamic process, which is of special importance when discussing the effects of dexrazoxane and amifostine on that feature.

The current results confirm that dexrazoxane is a more potent cardioprotector than amifostine (18). Far more important is the difference in their ability to reverse the progression of changes toward dilative cardiomyopathy. In the dexrazoxane-treated group, the grade of pathological changes remained the same between 1.5 and 3 months. Thus, dexrazoxane could easily be considered the agent that completely prevents further progression of histological changes. Considering that apoptotic markers were not followed during this investigation, we can only suggest that apoptosis or/and necrosis certainly occurred as a natural ending of affected cell existence (34–36). However, the same scores at both time point in the dexrazoxane group could indicate that the primary lesion was literally “frozen” for another 3 months, which is at least possible. Additionally, the complete absence of primary lesion spreading in time, in the presence of cell deaths, would lead to gradual disappearance of changed myocytes and consequent loss of any evidence of doxorubicin-specific alteration. According to all noted above, the single logical conclusion is that dexrazoxane administered prior to doxorubicin resulted in significant slowing down of myocardial devastation to the extent that it could not be termed as a progression. On the contrary, amifostine did not suspend primary lesion spreading. It offered protection to cardiac tissue against doxorubicin toxicity and restricted the size of the primary lesion, which can be concluded from individual scores at 1.5 months. However, comparing scores from 1.5 with scores 3 months after the treatment and these to scores in the doxorubicin-only–treated group, it seems that the progression rate in the amifostine-pretreated group was even accelerated.

Even though changes in the serum activity of many enzymes have already been used as markers, validation studies did not reveal that any of them was a confident early marker for doxorubicin cardiotoxicity. The increase of cardiac troponin T (cTnT) seemed to be the most sensitive when a third-generation assay was used (37). However, previous reports gave conflicting data for the relationship between the time of chemotherapy administration and the occurrence of pathologic levels of troponins (38, 39). Additional investigations revealed sufficient specificity, but gave no convincing evidence of cTnT sensitivity, making it suitable as an early detector, but not as a predictor of myocardial deterioration (40, 41).

Previously, we discussed changes in the activity of conventional serum markers in mice under doxorubicin-only treatment, with or without cardioprotector (21). Although no statistical method was used to confirm or deny the correlation between the alterations in serum marker activity and the results of a light microscopy examination, it was clear that the significant reduction in CK and α-HBDH leakage in the protected group compared to the doxorubicin-only treatment corresponded to favorable results of the histopathology score. Moreover, when those data are compared to the current results, two types of correlations between the results of biochemical analysis expressed as %difference and histological analysis can be observed. First, decrease in α-HBDH leakage is related to a reduction in the size of the primary lesion. Dexrazoxane achieved the greatest reduction of α-HBDH leakage compared to amifostine and vitamin E (21). Also, dexrazoxane provided the best protection of the heart tissue in comparison with two other protectors that induced an almost equal decrease in the activity of this serum marker and gained the same median histopathology score at 1.5 months. Secondly, considerably greater reduction of the CK leakage in reference to reduction of α-HBDH seems to be related to limited spreading of the pathologic process over time, which was established after the use of vitamin E (21) and dexrazoxane. Given that the accurate values of CK and α-HBDH %difference were quite different between those two groups, CK/α-HBDH %difference ratios were very similar—1.47 in dexrazoxane and 1.30 in vitamin E group. Although this observation cannot not be turned into a solid conclusion before more detailed studies, it can be concluded that the set of serum markers analyzed might be more helpful than a single one in predicting cardiomyopathy grade induced by the anthracycline therapy, as well as the overall effect of cardioprotective treatments.

The results of this study challenge previous assumptions that doxorubicin administration damages a number of heart muscle cells, which further increases only after the next doxorubicin treatment, whereas the delay in the occurrence of CHF relies only upon physiological compensation mechanisms. The experimental model used here is at variance with a clinical situation, but it yields information on a certain plasticity in the development of doxorubicin cardiomyopathy. Demonstrated dexrazoxane ability to reverse the evolution toward congestive heart failure strongly encourages its application, and respecting such evolution characteristics, suggests further investigations to revise dexrazoxane dosing schedules in order to minimize side effects (13) and maximize the cardioprotective effects. This investigation provides a fresh perspective that can help in discovering novel strategies to reduce the risk of doxorubicin-induced heart failure.

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