Protein Profiling in Daunorubicin-Induced Cardiomyopathy

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Abstract. The aim of this paper was to study the protein remodelling of the left ventricle following repeated administration of either daunorubicin (DNR) or DNR in combination with the cardioprotective agent dexrazoxane (DXZ). The experiment was carried out on three groups of Chinchilla male rabbits: 1. DNR (3 mg/kg i.v.), 2. DNR (3 mg/kg i.v.) + DXZ (60 mg/kg i.p.), and 3. the control group (saline 1 ml/kg i.v. in the same schedule). The drugs were given once weekly, max. 10 administrations. Protein fractions were isolated by stepwise extraction from the samples of the left ventricle. In the DNR-group, the concentrations of both, metabolic and contractile proteins were significantly reduced, while the amount of collagen was significantly higher in comparison with the control group. In the group treated with DNR and DXZ, the concentrations of individual protein fractions (except metabolic proteins) were comparable to those of the control group, which confirms a significant cardioprotective effect of DXZ. The changes of protein profiling corresponded to functional examination of both cardiac parameters (EF, $\mathrm{d}P/\mathrm{d}t_{\max}$, PEP : LVET index) and histological examination. These data should be used in further studies dealing with evaluation of cardiotoxic and, possibly, cardioprotective effects of new drugs.

Key words: Daunorubicin — Dexrazoxane — Cardiomyopathy — Protein profile — Collagen

Introduction

Treatment with anthracycline antibiotics may be associated with various types of cardiotoxicity (Ferrans et al. 1997): 1. acute toxicity is preferentially related to

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rapid i.v. administration and is manifested by hypotension and cardiac dysrhythmias; 2. subacute toxicity is uncommon, it develops early in the course of the therapy and is characterized by myocarditis and pericarditis; 3. chronic toxicity is the most common form of anthracycline-induced cardiotoxicity and is manifested by chronic dilated cardiomyopathy that develops late in the course of therapy or shortly after its termination; and 4. delayed toxicity is characterized by restrictive cardiomyopathy that develops at periods of time ranging from 10 to 15 years after the termination of treatment.

Long-term intravenous administration of daunorubicin (DNR) in rabbit (used in our study) induces signs of chronic cardiotoxicity, and the rabbit model is thus considered to be a satisfactory animal model for anthracycline-induced cardiomyopathy (Geršl and Hrdina 1994). A principal role in pathogenesis is possibly exerted by free oxygen radicals generated by "redox-cycling" of the anthracycline molecule, and/or by the formation of anthracycline-ferric ion complexes (Hrdina et al. 2000). At present, the only clinically approved drug for the prophylaxis of anthracycline-induced cardiotoxicity is a derivative of EDTA – dexrazoxane (DXZ) (Weiss et al. 1999). The mechanism of its cardioprotective activity is connected with intracellular chelation of ions, including Fe ions, that results in slowing down the formation of free oxygen radicals induced by anthracyclines (Link et al. 1996).

The aim of this paper was to study the changes in the protein profile of the left ventricle following repeated administration of DNR alone or in combination with the cardioprotective agent DXZ.

Materials and Methods

All experiments used in this study were approved by the Ethical Committee of the Charles University, Faculty of Medicine in Hradec Králové. The study was carried out in three groups of Medium size Chinchilla male rabbits (average weight 3 kg at the beginning of the experiment):

- control (saline 1 ml/kg i.v.), once a week, 10 administrations, $n = 10$;
- DNR (Cérubidine, Bellon Rhone-Poulenc, France, 3 mg/kg i.v.), once a week, 10 administrations, $n = 11$;
- DXZ (Cardioxane, Chiron, Netherlands, 60 mg/kg i.p. – 30 min before the administration of daunorubicin) + DNR (Cérubidine, Bellon Rhone-Poulenc, France, 3 mg/kg i.v.) once a week, 10 administrations, $n = 5$.

Determination of left ventricle protein profile

The metabolic, contractile and pepsin-soluble and insoluble collagenous proteins were isolated by stepwise extraction from the samples of the left ventricle (LV) by the method developed by Pelouch et al. (1995). The separation of individual fractions was confirmed using 12 % SDS-PAGE. Protein concentration was determined according to Lowry et al. (1951) and expressed per g of tissue wet weight. Hydroxyproline concentration was estimated in both collagenous fractions (Pelouch et al.

1993). A factor of 7.46 was used to convert the total concentration of hydroxyproline to the concentration of collagen.

Functional parameters of the left ventricle

Ejection fraction (EF) was determined using Vingmed CFM 800A (GE Medical Systems). Both non-invasive and invasive measurements were performed using ADI PowerLab/8SP (Australia) with the software Chart for Windows (v. 3.4.11). Phonocardiogram recordings, carotid pulse waveforms and standard limb leads for ECG detection were recorded and the index pre-ejection period : left ventricular ejection time (PEP : LVET) was calculated as a parameter of the function of the heart; haemodynamic measurement (LV $\mathrm{d}P/\mathrm{d}t_{\max}$) was performed at the end of experiment.

Histological examination

Histological examination after autopsy was performed using light microscopy. Tissue blocks of transversely sectioned LV (the region under the atria) were fixed by immersion in 10 % formalin. Paraffin sections (7 μm) were stained with haematoxylin-eosin and Masson's blue trichrome. A 6-point scale of morphological changes was created for histological evaluation in relation to the routinely described histopathological changes in damaged myocardium (Woolf 1992).

Statistical analysis

The values of individual parameters expressed in Figures and Tables are given as mean ± S.E.M. Statistical evaluation was performed using the SigmaStat software (Version 2.0 for MS Windows 95, Jandel GmbH, Erkrath, Germany).

Results

Protein profiling of the left cardiac ventricle

The concentration of total protein was significantly ($p < 0.05$) lower in the DNR-group (193.43 ± 9.72 mg/g wet weight) compared with both the control group (263.73 ± 11.30 mg/g wet weight) and the group in which a combination of DNR with DXZ was applied (249.22 ± 2.51 mg/g wet weight) (Fig. 1).

In more detail, the concentration in the fraction of metabolic proteins in the rabbit LVs following repeated administration of DNR (32.69 ± 2.68 mg/g wet weight) was significantly ($p < 0.05$) lower compared to the control group (62.43 ± 7.30 mg/g wet weight). The concentration of metabolic proteins was found to be 49.47 ± 3.05 mg/g wet weight in the group that obtained the cardioprotective agent DXZ (Fig. 2). Further, the concentration of contractile proteins in the DNR-group (55.07 ± 4.98 mg/g wet weight) was significantly ($p < 0.05$) reduced compared to both the control group (82.93 ± 5.65 mg/g wet weight) and to the group that received the DXZ-DNR combination (79.39 ± 3.20 mg/g wet weight) (Fig. 3). On the other hand, the amount of collagen in both soluble and insoluble isolated

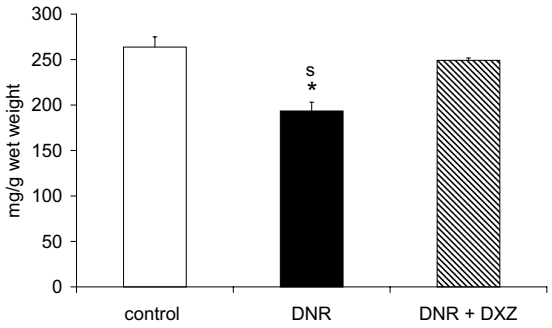

Figure 1. Concentration of total protein. * statistical significance ($p < 0.05$) in comparison with the control group; S, statistical significance ($p < 0.05$) in comparison with the dexrazoxane-daunorubicin (DXZ-DNR) combination.

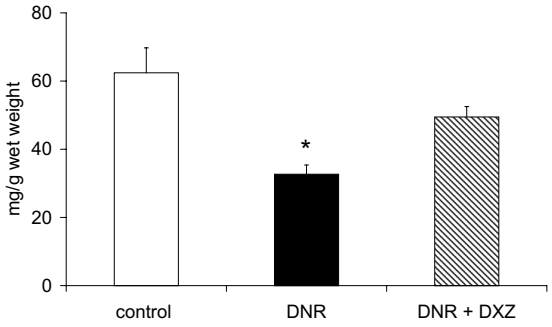

Figure 2. Protein profiling of metabolic proteins. * statistical significance ($p < 0.05$) in comparison with the control group.

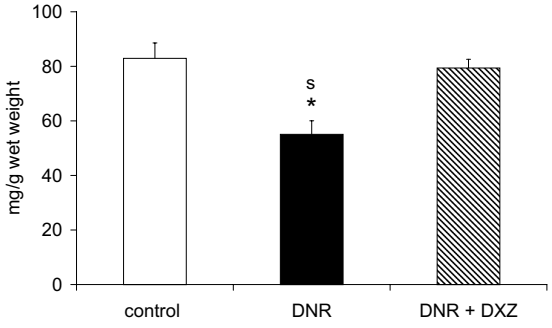

Figure 3. Protein profiling of contractile proteins. * statistical significance ($p < 0.05$) in comparison with the control group; S, statistical significance ($p < 0.05$) in comparison with the DXZ-DNR combination.

collagenous proteins was significantly higher in the DNR-group (13.39 ± 0.97 mg/g wet weight) as compared to both, the control group (10.03 ± 0.65 mg/g wet weight) and the group that received the DXZ-DNR combination (7.68 ± 0.65 mg/g wet weight) (Fig. 4).

Functional examination of the heart

The EF in the DNR-group was significantly ($p < 0.05$) decreased to the value 46.6

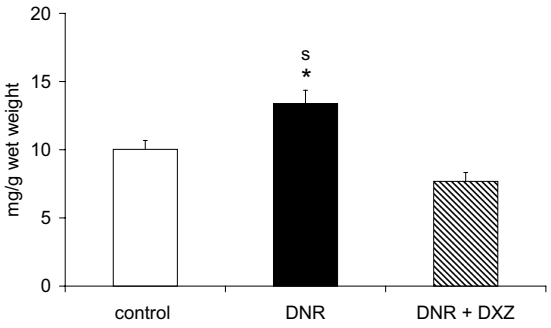

Figure 4. Protein profiling of total collagen. * statistical significance ($p < 0.05$) in comparison with the control group; S, statistical significance ($p < 0.05$) in comparison with the DXZ-DNR combination.

$\pm$ 3.0% (the initial value of the LVEF was 64.3 $\pm$ 2.0%) in comparison to both, the control group (the initial value 62.4 $\pm$ 1.0%) and to the group that received the DXZ-DNR combination (the initial value 63.0 $\pm$ 1.5%). Furthermore, the administration of DNR induced a significant, progressive increase in the PEP : LVET index of about 30% (the initial absolute value was 0.407 $\pm$ 0.016) in comparison to the control group (0.430 $\pm$ 0.008) and to the group treated with DNR and DXZ (0.404 $\pm$ 0.031). Invasive haemodynamic measurement at the end of the experiment also confirmed a significant decrease in $\mathrm{d}P/\mathrm{d}t_{\max}$ in the DNR-group (5594 $\pm$ 520 mm Hg$\cdot$s^{-1}) compared to the control group (9645 $\pm$ 790 mm Hg$\cdot$s^{-1}) and to the group that received the combination of DNR with DXZ (10,585 $\pm$ 581 mm Hg$\cdot$s^{-1}). The functional parameters of the LV at the end of experiment are summarized in Table 1.

Histological examination of the left ventricle

In the DNR-group, diffuse toxic damage was found in the whole myocardium (maximal in the ventral part of the LV). The morphological picture demonstrated that, after the administration of DNR, diffuse cytolysis of cardiomyocytes developed, followed by reparative changes resulting in interstitial fibrosis. On the other hand, only moderate morphological changes in the myocardium were observed after administration of DNR with DXZ. The changes were characterized by a score of 1,

Table 1. Functional examination of the LV at the end of experiment

	Control	DNR	DNR + DXZ
PEP : LVET	0.427 $\pm$ 0.019	0.531 $\pm$ 0.026*S	0.414 $\pm$ 0.044
LVEF (%)	61.6 $\pm$ 1.0	46.6 $\pm$ 3.0*S	56.8 $\pm$ 3.0
$\mathrm{d}P/\mathrm{d}t_{\max}$ (mm Hg$\cdot$s^{-1})	9 645 $\pm$ 790	5 594 $\pm$ 520*S	10 585 $\pm$ 581

PEP : LVET, pre-ejection period : left ventricular ejection time; LVEF, ejection fraction of the left ventricle; * statistical significance ($p < 0.05$) in comparison with the control group; S, statistical significance ($p < 0.05$) in comparison with the dexrazoxane-daunorubicin (DXZ-DNR) combination.

Table 2. Evaluation of morphological changes in the myocardium of LV

		Degree of changes and its incidence						
	n	0	1	2	3	4	5	6
Control	10	9	1	0	0	0	0	0
DNR	11	0	1	1	0	5	4	0
DNR + DXZ	5	2	3	0	0	0	0	0

Score 0, normal appearance of the tissue; Score 1, the changes of tinction (increased eosinophilia of the cytoplasm); Score 2, scattered groups of degenerated myocytes; Score 3, numerous foci of degenerated myocytes, but yet without myofibrosis; Score 4, degenerated cells are replaced by proliferation of interstitial connective tissue; Score 5, the changes in larger amount as in previous score; Score 6, very conspicuous, disperse toxic injury of myocardium with subsequent interstitial fibrosis (the scars sometimes transmural).

i.e., some smaller or larger spots of degenerated myocytes were dispersed in the myocardium of entirely normal structure. The myocytes forming these foci were mostly of homogenous, intensely eosinophilic cytoplasm resulting from fragmentation and breakdown of myofibrils; only few myocytes were necrotic, marked by the presence of macrophages. Evaluation of the morphological changes in the myocardium of LV using the 6-point scale is summarized in Table 2.

Discussion

The protein profile procedure yielded three basic fractions: 1. metabolic proteins (containing predominantly enzyme systems for aerobic and anaerobic substrate utilization); 2. contractile proteins (a complex of contractile, regulatory and modulatory proteins of the myofibrils); 3. structural collagenous proteins (the fraction included collagens, elastins, proteoglycans and glycoproteins) that can be divided into two fractions: a) soluble collagenous proteins constituted mainly by collagen I and III, and b) insoluble collagenous proteins including collagen aggregates, elastins and other proteins of the extracellular matrix (Pelouch et al. 1995).

The application of DNR brought about remodelling of the protein composition of cardiac muscle; it was predominantly characterised by a lower concentration of total protein in the DNR-group that reflects mainly a decreased concentration of non-collagenous proteins, i.e., both metabolic and contractile proteins. A second important factor contributing to the reduced concentration of the total protein is interstitial oedema (Ferrans 1978), in accordance with the observations showing that cardiac tissue in anthracycline-induced toxicity has an increased content of water, sodium and calcium (Olson et al. 1974). The reduced concentration of both metabolic and contractile proteins is of crucial importance in the pathogenesis of anthracycline-induced cardiomyopathy and results at first from inhibition of protein synthesis, and later from the loss of cardiomyocytes (Ferrans 1978). Inhibition of nucleic acid and protein synthesis is caused by the intercalation of the drug

into nuclear DNA (Yang and Wang 1999). This mechanism of DNR toxicity is especially pronounced in myofibrils of cardiac muscle, since the half-life of their contractile proteins is relatively short (Gulve et al. 1991). Later, degeneration of cardiac muscle cells can assume two forms: necrosis (could be detected by the analysis of extracellular matrix proteins – e.g., by collagen concentration) and apoptosis (Laurent and Jaffrezou 2001). However, the relative role of cardiomyocyte necrosis and apoptosis during development of anthracycline-induced cardiomyopathy has not been evaluated. Several authors demonstrated that in patients with heart failure the level of necrosis was 7-times larger than that of apoptosis (Schaper et al. 1999). Our previous study showed that release of cardiac troponin T into circulation is a very sensitive predictive marker of anthracycline-induced cardiomyopathy in rabbits (Adamcová et al. 1999).

The interstitial fibrosis is difficult to evaluate because cellular degeneration exaggerates the prominence of the interstitial connective tissue. It is believed that activation of metalloproteinases (enzymes destroying the extracellular matrix) and reduction of tissue inhibitors of metalloproteinases triggered predominantly by the oxygen free radicals presents the initial step of myocardial remodelling of extracellular matrix during adaptive response of the heart to the action of anthracyclines (Kapelko 2001). It brings about the destruction of some collagen links with further increased synthesis of collagen of types III and I. The significant alteration of the collagen network creates a basis for the development of fibrosis accompanying dilated cardiomyopathy. Our data demonstrated an increased concentration of total collagen in the DNR-group in comparison with both, the control group and the group receiving DNR in combination with DXZ. The increased concentration of hydroxyproline dominated mainly in the soluble collagenous fraction, where newly synthesized forms of collagen were recovered.

Further it is necessary to take into account that the biosynthesis of collagen can also be affected by DNR. One of the recognized side effects accompanying anthracycline administration is poor wound healing resulting from impairment of collagen biosynthesis. Although the precise mechanism has not been established, some authors suggested that prolidase, an enzyme involved in collagen metabolism, may be one of the targets for anthracycline-induced inhibition of synthesis of this protein (Muszynska et al. 2001).

Our biochemical data reflect the chronic cardiotoxicity of anthracycline characterized by progressive cardiomyopathy with myocyte degeneration, interstitial oedema and fibroplasia leading to fibrosis. Unlike the diffuse toxic damage of the LV, the extent of damage of the right ventricle wall is always markedly smaller. The changes of protein profiling corresponded to functional examination of the LV. The decreased concentration of both metabolic and contractile proteins, and the increased content of collagen after long-term i.v. administration of DNR were accompanied by a significant increase of the PEP : LVET ratio and by a reduction of both the EF and the index $\mathrm{d}P/\mathrm{d}t_{\max}$.

In the group treated with DNR and DXZ the concentrations of individual protein fractions were comparable to those of the control group, which confirms a

significant cardioprotective effect of DXZ. Although DXZ is usually reported not to affect the antitumour activity of anthracyclines and not to increase the incidence of non-cardiac toxicities (Wexler et al. 1996), it was found that DXZ in higher doses (>600–750 mg/m^2 i.v.) may increase bone marrow depression (Hochster et al. 1992). This finding encourages the investigation of other potential cardioprotectants with low toxicity.

It is possible to conclude that we have evaluated the protein profiling in the control and two reference groups of animals. These data should be used in further studies dealing with evaluation of cardiotoxic and, possibly, cardioprotective effects of new drugs.

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