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Myocardial content of selected elements in experimental anthracycline-induced cardiomyopathy in rabbits

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Abstract

Cardiotoxicity represents the main drawback of clinical usefulness of anthracycline antineoplastic drugs. In this study, a content of selected elements (Ca, Mg, K, Se, Fe) in the post-mortem removed samples of the myocardial tissue was studied in three groups of rabbits: 1) control group (i.v. saline; $n = 10$); 2) daunorubicin-receiving animals (DAU; 3 mg/kg, i.v; $n = 11$); 3) animals receiving cardioprotective iron-chelating agent dexrazoxane (DEX; 60 mg/kg, i.p.; $n = 5$) prior to DAU. Drugs were administered once weekly for 10 weeks. 5–7 days after the last administration, cardiac left ventricular contractility (dP/dt_{max}) was significantly decreased in DAU-treated animals (745 ± 69 *versus* 1245 ± 86 kPa/s in the control group; $P < 0.05$), while in the DEX + DAU group it was insignificantly increased (1411 ± 77 kPa/s). Of the myocardial elements' content studied, a significant increase in total Ca against control (16.2 ± 2.4 *versus* 10.6 ± 0.9 μg/g of dry tissue; $P < 0.05$) was determined in the DAU-group, which was accompanied with significant decreases in Mg and K. In the heart tissue of DEX-pretreated animals, no significant changes of elements' content were found as compared to controls, while the Ca content was in these animals significantly lower than in the DAU group (9.1 ± 0.4 *versus* 16.2 ± 2.4 μg/g; $P < 0.05$). Hence, in this study we show that systolic heart failure induced by chronic DAU administration is primarily accompanied by persistent calcium overload of cardiac tissue and the protective action of DEX is associated with the restoration of normal myocardial Ca content.

Introduction

Anthracycline (ANT) antineoplastic antibiotics (e.g., doxorubicin and daunorubicin) rank among the most effective anticancer drugs ever developed (Calabresi & Chabner 2001). Cardiotoxicity, however, represents a serious adverse effect that continues to limit their therapeutic potential and threatens the cardiac function of many patients with cancer. The main risk of anthracyclines is associated with their chronic administration, when severe cardiomyopathy and congestive heart failure may develop any time after the completion of the treatment (Shan *et al.* 1996; Hrdina *et al.* 2000).

The precise pathogenesis of anthracycline-induced cardiotoxicity is still uncertain, and it is most likely of multifactorial origin (Minotti *et al.* 2004). Nevertheless, pivotal role is attributed to the iron-catalyzed intramyocardial production of reactive oxygen species (ROS), which cause damage of various targets in the myocardial cells (Doroshow 1983; Rajagopalan *et al.* 1988). Free cellular iron has been shown to participate in the ROS production, both as a catalyst of the hydroxyl

radicals' production (via the Haber–Weiss reaction) and by forming the ANT–Fe complexes (Olson & Mushlin 1990; Gille *et al*. 1997). The importance of iron in the ethiopathogenesis of anthracycline-induced cardiotoxicity has been confirmed by the high protective efficiency of dexrazoxane (ICRF-187) – the only clinically approved cardioprotectant so far (Wiseman *et al*. 1998; Swain *et al*. 2004). Dexrazoxane apparently protects cardiomyocytes against anthracycline-induced damage through its potent metal-chelating hydrolysis product ADR-925, which acts by displacing iron bound to anthracycline or chelating free or loosely bound iron and thus preventing the iron-based ROS damage (Buss *et al*. 1993; Hasinoff *et al*. 1998). Importantly, iron chelation has also been shown to possess antiproliferative effects against malignant cells (Richardson 1997) and the Fe chelating cardioprotective strategy can thus also result in enhanced antitumour activity (Kwok & Richardson 2000).

In rabbit, chronic administration of anthracyclines causes reproducible cardiac damage, similar to those observed in humans, and the rabbit is thus considered to be a satisfactory animal model for experimental anthracycline cardiomyopathy induction (Herman & Ferrans 1998). Choice of daunorubicin as a model anthracycline as well as its dosage schedule was based upon our previous studies (Geršl & Hrdina 1994; Klimtová *et al*. 2002).

The aim of this study was to experimentally induce the anthracycline cardiomyopathy in rabbits, with subsequent analysis of the myocardial content of selected elements (Ca, Mg, K, Se, Fe) and their correlations. Furthermore, the effects of concurrent cardioprotective administration of dexrazoxane were investigated.

Methods

Study design

Medium size Chinchilla male rabbits with average body weight 3.3 kg at the beginning of the experiment were used. The animals were maintained in an air-conditioned room, allowed free access to a standard pellet rabbit diet and tap water. The study was performed under the supervision of the Ethical Committee of the Charles University in Prague, Faculty of Medicine in Hradec Králové, and it conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1985).

Three groups of experimental animals were used:

1. Control group (10 animals) received saline (1 ml/kg, i.v.)
2. DAU group (11 animals) received daunorubicin (Cèrubidine, Bellon Rhône-Poulenc Rorer, France – 3 mg/kg – i.e. approximately 50 mg/m^2, i.v.)
3. DEX + DAU group (5 animals) received dexrazoxane (Cardioxane, Chiron B.V., The Netherlands – 60 mg/kg, i.p.), 30 min prior to daunorubicin (3 mg/kg, i.v.)

All substances were administered once weekly for 10 weeks. 5–7 days after the last administration, invasive contractility measurements had been performed in surviving animals which were then sacrificed with pentobarbitone overdose. Samples of the left ventricular myocardial tissue were then immediately removed for determinations of elements' content.

Determination of myocardial content of selected elements

Content of calcium (Ca), magnesium (Mg), potassium (K), selenium (Se) and iron (Fe) was measured in samples of the left ventricular myocardium. Samples were dried and after cooling period they were weighed and digested by microwave digestion with nitric acid and hydrogen peroxide. Magnesium, iron and selenium were determined using graphite furnace atomic absorption spectrometry (Unicam, Solaar 959, U.K.). Calcium and potassium were measured photometrically using flame photometry (Eppendorf, Efox 5053, Germany). Results are expressed as ng/g of dry tissue, selenium as μg/g of dry tissue.

Left ventricular contractility measurement

In pentobarbitone anaesthesia (Nembutal Sodium inj., Abbott, U.S.A. – 30 mg/kg i.v.), a polyethylene catheter filled with heparinized (10 IU/ml) saline was introduced via the left carotide artery into the left heart ventricle. Maximal rate (maximum of the first derivative) of the pressure rise in

the isovolumic phase of the systole – dP/dt_{max} was determined as an index of the left ventricular contractile function, using ADI PowerLab/8SP (Adinstruments, Australia).

Statistical analysis

Data are expressed as means ± S.E.M. The statistical software SigmaStat for Windows 2.0 (Jandel, Germany) was used in this study. Significance of differences between groups was estimated using One Way ANOVA unpaired test or Kruskal-Wallis ANOVA on Ranks (data without normal distribution). Pearson correlation analysis was used to describe the relations between the variables. $P < 0.05$ was used as a level of statistical significance, unless indicated otherwise.

Results

Mortality

In the DAU group, four animals of 11 (37%) died or were moribund and had to be sacrificed prematurely. In the other two groups (control and DEX + DAU) no premature deaths occurred.

Left ventricular contractility

As seen in Figure 1, left ventricular contractility was in 7 surviving DAU-treated animals significantly reduced to 59.8% of the control values. In dexrazoxane-pretreated rabbits, the dP/dt_{max} values did not differ significantly from control and were significantly higher than in the DAU-group.

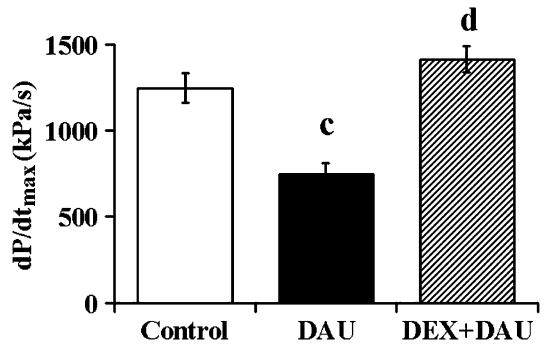

Figure 1. Left ventricular contractility after the 10-week treatment with daunorubicin alone (DAU) or in a combination with dexrazoxane (DEX + DAU). dP/dt_{max} – maximal rate of the pressure rise in the isovolumic phase of the systole. Statistical significance (ANOVA, $P < 0.05$): c – comparison with control group, d – comparison with DAU group.

Myocardial content of selected elements

Results are summarized in Table 1. In the DAU group, a significant increase in total myocardial calcium and decrease in magnesium and potassium contents were detected. In animals, where dexrazoxane was administered prior to daunorubicin, no significant changes were found as compared to control group, while calcium content was in these animals significantly lower than in the DAU group.

Correlation analyses

In order to assess potential connections between the left ventricular contractility and the elements' content as well as the interrelationships among the elements, Pearson correlation analysis was performed. Of the 15 pairs of variables tested, those with statistically significant correlation are listed in Table 2. Regarding the cardiac contractility, negative correlation was detected with calcium content (Figure 2). Furthermore left ventricular Ca content also correlated with Mg and Se (negative and positive correlations, resp.). The strongest positive correlation was observed between the contents of Mg and K (Figure 3).

Discussion

Changes in various ionic concentrations and their ratios in cardiac cells are known to be important features that accompany heart failure, including that resulting from anthracycline-induced cardiomyopathy. While there have been performed numerous *in vitro* studies with isolated cardiac preparations or cells and mostly dealing with acute effects of anthracyclines, we have carried out a whole-animal study, with repeated administration of the daunorubicin and subsequent determination of various elements' content in the post-mortem removed samples of the left ventricular tissue. Furthermore, effects of pre-treatment with dexrazoxane, a well established cardioprotective agent, were studied. We assume that our experimental model can mimic changes in human

Table 1. Myocardial content of selected elements.

	Calcium (μg/g)	Magnesium (μg/g)	Potassium (μg/g)	Iron (μg/g)	Selenium (ng/g)
Control	10.6 ± 0.86	31.8 ± 0.52	249 ± 5	2.92 ± 0.21	7.31 ± 1.40
Daunorubicin	16.2 ± 2.36 c	28.6 ± 0.91 c	225 ± 5 c	2.84 ± 0.24	10.94 ± 2.55
Daunorubicin + dexrazoxane	9.06 ± 0.42 d	29.7 ± 0.87	227 ± 14	2.47 ± 0.08	8.26 ± 0.46

Results are expressed per gram of dry left ventricular tissue. Statistical significance (ANOVA, $P < 0.05$): c – comparison with control group, d – comparison with daunorubicin group.

Table 2. Variables with statistically significant correlations, given as Pearson's correlation coefficients.

Variables	Correlation coefficient	Significance
dP/dt_{max}/Ca	−0.60	$P < 0.01$
Ca/Mg	−0.42	$P < 0.05$
Ca/Se	0.59	$P < 0.005$
Mg/K	0.73	$P < 0.0001$

myocardium that accompany the heart failure development after the chronic anthracycline-containing chemotherapy as well as the protection against this cardiotoxicity with dexrazoxane. The period between the last administration and performed measurements was intended to allow elimination of the drugs from the organism. Measured values of both contractility and the content of elements thus reflect the long-term myocardial changes, which persist after the wash-out of daunorubicin and dexrazoxane, rather than their acute effects.

Repeated 10-week DAU administration (in a cummulative dose of 30 mg/kg, i.e., approximately 500 mg/m^2) resulted in premature death of 37% of animals as well as in significant left ventricular contractility reduction, indicating the heart failure development. Administration of dexrazoxane 30 min before each DAU application was associated with 0% mortality and complete preservation of the contractility, further confirming its high cardioprotective efficiency found in different animal models (Imondi *et al.* 1996; Herman & Ferrans 1998).

As shown in our previous paper (Adamcova *et al.* 2003), repeated daunorubicin administration results in rabbits in pronounced remodelling of the protein composition of cardiac muscle, where the concentrations of both metabolic and contractile proteins are significantly reduced, while the amount of collagen is significantly higher in comparison with control group. In the dexrazoxane pre-treated rabbits, the concentrations of individual protein fractions were shown to be mostly comparable to those of the control group.

Regarding the myocardial content of studied elements, in the present study, the most pronounced difference between the control and DAU groups was observed in the content of calcium – a significant increase to 153% of the control values

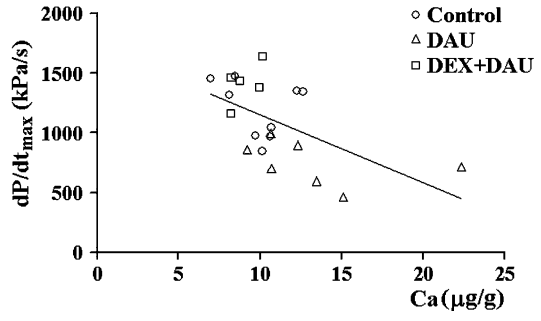

Figure 2. Scatterplot of the left ventricular contractility (dP/dt_{max}) versus post-mortem-determined left ventricular total calcium content. LV contractility was measured out immediately before the killing of animals and taking samples of the cardiac tissue for elements' content analysis.

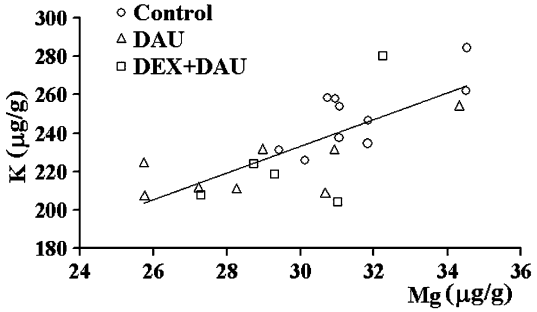

Figure 3. Scatterplot of the post-mortem-determined left ventricular total magnesium and potassium contents.

was noticed in the daunorubicin-receiving group. Abnormal myocardial calcium handling, usually manifested as increased calcium concentrations in cytosol and known as 'calcium overload of cardiac cells', is reported to be one of the most important features of anthracycline-induced cardiomyopathy. Anthracyclines have been shown to activate the calcium release from sarcoplasmatic reticulum (Holmberg & Williams 1990; Pessah *et al.* 1990) as well as they may interfere with its accumulation (Halili-Rutman *et al.* 1997). This results in a decrease in the amplitude of Ca^{2+} transients caused by an increased diastolic and decreased peak systolic Ca^{2+} concentrations (Temma *et al.* 1997). The cardiomyocytes' calcium overload leads to mitochondrial function disturbances, ATP depletion and contractility impairment. In our study, decreased left ventricular contractility in daunorubicin-receiving rabbits was found to negatively correlate with increased calcium left ventricular content, which confirms the above discussed findings, obtained from *in vitro* experiments. Dexrazoxane, whose cardioprotective effect is attributed to iron chelating action of its hydrolysis product ADR-925 and thus oxidative stress reduction, has been shown to normalize the Ca content (Table 1). Indeed, the 'free-radical' and 'calcium overload' putative pathways of anthracycline-induced cardiotoxicity seem to go hand in hand as numerous papers have shown the link between the cellular ROS imbalance and calcium homeostasis disturbances (Burton *et al.* 1990; Holmberg *et al.* 1991).

Distinct increase in total calcium was in the myocardium of DAU group of rabbits accompanied with less pronounced, though statistically significant decreases in magnesium and potassium contents. Indeed, Mg is known to be closely linked with various abnormalities that accompany heart failure: it is important for operation of the Na^+/K^+ pump, responsible for electrochemical gradient across the cytoplasmic membrane, and Mg is an important co-factor of many enzymatic cellular reactions involved in the energetic metabolism (Saris *et al.* 2000; Delva 2003). Magnesium concentration is also known to influence the sarcoplasmatic reticulum to sequester calcium (Chiesi & Inesi 1981). Decrease in myocardial Mg content has been shown in experimental heart failure induced by rapid ventricular pacing in dogs (Haigney *et al.* 1998), as well as in the biopsies from heart failure patients (Ralston *et al.* 1989). Regarding the anthracycline-induced cardiotoxicity, in a clinical study by Sartori *et al.* (1991), intracellular Mg content decreases paralleled the severity of cardiac disturbances in patients receiving doxorubicin or epirubicin-containing chemotherapy. The mechanism causing this cardiac Mg loss is unknown. Romani & Scarpa (1992) reported an efflux of 10% to 15% of total cellular magnesium within 10 min from isolated rat hearts in response to 10 μmol/L norepinephrine, which suggests that neurohumoral activation in heart failure may result in the loss of cardiac Mg.

Intracellular magnesium is known to produce a block of the outward movement of K^+ (Ishihara *et al.* 1989), so the reduction of intracellular magnesium may favor the efflux of potassium from the cells. Intracellular Mg is also able to block the efflux of K^+ through a channel known as ATP-dependent K^+ channel (Horie *et al.* 1987). By analogy, intracellular Mg reduction favors the efflux of K^+ also through this channel. The modulating action of Mg on both the above-mentioned channels would explain the reduction in cell potassium seen in our study as well as the Mg/K correlation, the strongest one seen in our study (Table 2, Figure 3). Similar strong direct Mg/K correlation was found in the tissues of heart failure patients studied by Ralston *et al.* (1989).

While the DAU-induced increase in total myocardial Ca content was completely prevented with dexrazoxazone pre-treatment, the decreases in myocardial Mg and K were affected with DEX only partially and insignificantly. As this DEX administration fully prevented contractility impairment, and the observed DAU-induced Mg and K content decreases do not correlate with contractility impairment, the Mg/K changes do not seem to play pivotal role in the DAU-induced heart failure.

Assuming the central role of iron in the pathophysiology of anthracycline-induced cardiotoxicity as well as in the dexrazoxane-afforded protection, myocardial content of Fe in both experimental groups was of our great interest. However, no significant changes were detected in either group. In a recent *in vitro* study, Kwok & Richardson (2003) showed 3–5 fold increased accumulation of Fe into ferritin in cardiomyocytes exposed acutely to various anthracyclines, apparently through the inhibition of the ferritin iron mobilization pathway. The present study, however,

does not indicate any longer-lasting significant myocardial Fe accumulation, as in our *in vivo* model of chronic anthracycline cardiotoxicity only slight and insignificant decrease could be observed. Similarly, dexrazoxane pre-treatment has resulted only in a statistically insignificant trend towards Fe content reduction. Reasons for the lack of more pronounced iron content decrease in dexrazoxane-pretreated group may include the fact that ADR-925 probably only shields free or loosely bound intramyocardial iron and prevents its participation in ROS production, rather than mobilizes it from the cells. Furthermore, the week interval since the last DAU or DEX + DAU administration could have permitted restoration of initial Fe levels.

A tendency to an increase in selenium concentration in daunorubicin group may reflect an up-regulation of glutathione peroxidase (GSX). GSX has been shown to serve as a major metabolic form of selenium (Cheng *et al*. 1998) and this enzyme has been reported to be strongly inducible by oxidative stress. This has been shown also in various models of anthracycline cardiotoxocity (Yin *et al*. 1998). DEX pre-treatment has partially attenuated this Se content increase, which may reflect oxidative stress reduction. However, as neither change reached the level of statistical significance, these findings have to be interpreted with a great caution.

In conclusion, in the present study, experimental cardiomyopathy and systolic heart failure were developed using repeated 10-week administration of daunorubicin. We have performed left ventricular contractility measurements, analysis of total myocardial content of selected elements and tried to study possible relations between those parameters. Our data support the hypothesis of crucial role of calcium overload as a hallmark of anthracycline-induced cardiotoxicity, which is mostly derived from *in vitro* experiments. Furthermore, disturbances of magnesium and potassium homeostasis were observed and those seem to be closely related. The dexrazoxane-afforded protection was associated with the restoration of normal Ca content.

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