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An Experimental Study on the Sarcoplasmic Reticulum Calcium Handling in Myocardium Intoxicated by Doxorubicin

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Abstract

Objective: Doxorubicin (DOX) is one of the most effective antineoplastic agents. However, the optimal clinical use of this agent is limited because of marked cardiomyopathy and congestive heart failure. This study was designed to explore the changes in the calcium pump protein or the calcium release channel of the sarcoplasmic reticulum during chronic doxorubicin treatment.

Methods: The rats were treated with intravenous doxorubicin (1.5 mg/kg) twice a week for 12 times. Controls received intravenous normal saline. The severity of cardiornyopathy was scored by light and electron microscopic study to investigate left ventricular papillary muscle and the calcium handling of the myocardial sarcoplasmic reticulum (SR) was determined using the isotope $Ca2^{+}$ loading.

Results: The ability of SR $Ca2^{+}$ uptake was decreased in doxorubicin-treated rats compared with control rats and the magnitude of the decrease in SR $Ca2^{+}$ uptake was correlated with the severity of the cardiomyopathy graded by pathology score.

Conclusion: The altered function of SR calcium uptake and release could lead to the abnormalities of contraction and relaxation observed in the doxorubicin cardiomyopathy.

Keywords: Doxorubicin; Cardiomyopathy; Sarcoplasmic reticulum; Calcium uptake; Sarcoplasmic reticulum Ca^{2+}-ATPase

Introduction

Doxorubicin is a highly effective cancer chemotherapeutic agent, but its clinical usefulness is limited due to the development of a dose-dependent cardiomyopathy [1]. Cancer chemotherapy with Doxorubicin (DOX) can cause severe cardiomyopathy thus leading to fatal congestive heart failure [2]. The total dose is usually limited to 450-500 mg/m^2 body surface area, since the incidence of the cardiomyopathy is "low" below this dose. However, more than half of the patients could tolerate higher total dose without development of cardiomyopathy, whereas a small percent of patients will develop the cardiomyopathy at even these low doses [3,4]. Endo-myocardial biopsy has been used to monitor the doxorubicin cardiomyopathy. Billingham [5] described the morphological changes seen on biopsy specimens from patients receiving doxorubicin. The earliest changes are distended sarcoplasmic reticulum and early myofibrillar loss. Later changes suggest diffuse cell damage with degeneration of multiple cellular organelles. These early morphologic abnormalities of the sarcoplasmic reticulum have been described in animal models as Well [6]. The DOX-induced cardiomyopathy is characterized by abnormal cytosolic concentration of Ca^{2+} [7-9]. In cardiomyocytes, Sarcoplasmic Reticulum (SR) determines cytosolic levels of Ca^{2+} via the ATP-dependent Ca^{2+}-pump mechanism [10]. The sarcoplasmic reticulum regulates the intracellular calcium stores on which adult mammalian cardiac muscle is dependent for contraction [11]. A number of studies have focused on the *in vitro* effects of anthracyclines on function of the pump and channel of this subcellular membrane system. Doxorubicin induces calcium release from isolated sarcoplasmic reticulum vesicles and in skinned cardiac fibers [12]. Doxorubicin binds to the calcium release channel in fractions enriched in terminal cisternae. Doxorubicin also increases opening probability of calcium release channels in reconstituted lipid bilayers [9]. Doxorubicinol, a metabolite of doxorubicin, is a potent inhibitor of multiple intracellular pumps, including the calcium pump protein of the cardiac SR in isolated SR vesicles. However, higher anthracycline concentrations appear to be required to mediate effects on the calcium-dependent ATPase than on the calcium release channel. Thus, there is compelling evidence that anthracyclines alter the function of the SR *in vitro*, suggesting that the contractile dysfunction of anthracycline-induced cardiomyopathy might be mediated by similar effects *in vivo*. The purpose of this study was to assess the effects of chronic doxorubicin administration on SR function and to correlate changes in SR function with functional and microscopic evidence of toxic cardiomyopathy.

Materials and Methods

Experimental model

All protocols were approved by Jiaotong Animal Care Committee of the University of Shanghai in accordance with the standards of the China Council on Animal Care. 60 healthy male Sprague-Dawley rats weighing 220-250 g were used in this study. Animals were kept under controlled conditions of temperature (22°C), relative humidity (55%), and 12 hour light/12 hour dark cycle. The animals were fed with standard chow, and tap water was supplied ad libitum.

Doxorubicin (Pharmacia, North Peapack, New Jersey, USA) was dissolved in sterile saline and administered intravenous in 12 equal

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injections, each containing 1.5 mg/kg, over a period of 6 weeks for a total cumulative dose of 18 mg/kg body weight [13].

Experimental design

Rats were divided into the following 2 groups. The sham control group comprised 20 animals and received standard chow diet for 6 weeks. The DOX-treated group comprised 40 animals: DOX was given intravenous at a dose of 1.5 mg/kg×12 times over a period of 6 weeks with a total dose of 18 mg/kg body weight [13].

Hemodynamic studies

The animals were anesthetized with intraperitoneal injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). The right carotid artery was exposed and cannulated with a microtip pressure transducer which was introduced via proximal arteriotomy. The catheter was carefully advanced through the lumen of the carotid artery until the tip of the transducer entered the left ventricle. The catheter was secured with a silk ligature around the artery allowing recordings of various hemodynamic parameters.

Echocardiographic measurements

Two-dimensional echocardiography was performed on rats lightly anesthetized with 0.5% halothane using Echocardio-Graphic system (SSD-5500; Aloka, Tokyo, Japan) equipped with a 7.5 MHz linear scan probe. The Left Ventricular End Diastolic Pressure (LVEDP), Left Ventricular Systolic Pressure (LVSP), and the rates of maximum pressure development and pressure fall (+dP/ dt and −dP/dt) were measured.

Cardiac enzymes in blood plasma

After 6 weeks of DOX treatment, blood samples were obtained. Plasma Lactate Dehydrogenase (LDH), Creatine Kinase (CPK), and Aspartate Amino Transferase (AST) were determined using commercial kits (Sigma chemical company). Concentrations of plasma troponin I (cTn I) and brain natriuretic peptide (BNP) were assessed using ELISA kits (Sigma, shanghai, China).

Light microscopy and electron micrograph EM scoring

Left ventricular papillary muscles were scored by a cardiovascular pathologist in a blinded manner. The severity of doxorubicin-induced myopathic changes was graded by light microscopy and confirmed by EM, according to the method of Billingham [5]. Overall scores for each rat was derived by examination of 5 to 10 specimens. To allow comparison of various parameters with degree of myopathy, the rats were arbitrarily subdivided into mild, moderate, and severe myopathy according to their microscopic score. The score of <1 was considered mild, 1~2 moderate, and >2 severe.

Isolation of SR membrane

Membrane fraction enriched in SR was isolated according to Ganguly et al. [14]. Briefly, viable left ventricular tissue from four to six hearts were homogenized in Waring blender in medium containing 10 mM $NaHCO_3$, 5 mM NaN_3, and 15 mM Tris–HCl (pH 6.8) at a moderate speed for 45 sec. The homogenate was then centrifuged at 10,000×g for 20 min. The resulting pellet was discarded, and the supernatant centrifuged again at 40,000×g for 30 min. The pellets obtained during the second centrifugation were resuspended in 600 mM KCl and 20 mM Tris–HCl (pH 6.8) to solubilize contractile proteins, and re-centrifuged at 40,000×g for 5 min. The final pellets were washed and resuspended in 250 mM sucrose and 20 mM Tris–HCl. Biochemical measurements were carried out using freshly prepared SR membranes. This SR preparation was relatively free from the mitochondria and contractile protein contamination. Membrane purity and protein concentration were determined as described elsewhere [15].

The SR Ca^{2+} uptake assay

The SR Ca^{2+} uptake activity was determined using the Millipore filtration technique [15]. Briefly, SR vesicles (0.03-0.08 mg/ml) were incubated in the presence of 100 mM KCl, 20 mM Tris–HCl (pH 6.8), 5 mM MgCl2, 5 mM K-oxalate, and 5 mM NaN3. The desired concentration of free $45Ca^{2+}$ was maintained by buffering 45CaCl2 solution (Sigma) with EGTA [16]. The reaction was initiated by adding 5 mM ATP. At the desired time, a 200 µl aliquot of the reaction mixture was filtered through Millipore filter (0.45 µM), after which the filter was immediately washed with 2 ml of ice-cold water. The filter was dried, and radioactivity was quantified using standard liquid scintillation counting technique. The ATP-independent Ca^{2+} uptake was determined in the absence of ATP, and this value was subtracted from the total Ca^{2+} uptake in the presence of ATP to obtain the ATP-dependent Ca^{2+} uptake activity.

Measurement of SR Ca^{2+}-stimulated ATPase activity

Basal and total activities of Ca^{2+}-stimulated ATPase were quantified in the incubation medium similar to the method used in the Ca^{2+} uptake assay [13]. Total ATPase was measured using non-radioactive CaCl2 (final concentration of free Ca^{2+}: 10 µM), while basal ATPase was measured in the absence of Ca^{2+} and in the presence of 0.2 mM EGTA. After a 5 min pre-incubation of the reaction mixture with 0.05 mg/ml of membrane, reaction was started by addition of 5 mM Tris - ATP and terminated with 12% ice-cold trichloroacetic acid. Inorganic phosphate liberated during this reaction was estimated in a protein-free filtrate by spectrophotometric method [14].

Statistical analysis

Statistical analysis was performed using SPSS 11.5 software. Quantitative data are expressed as mean ± SD. The t test was used for comparison between two groups, the p values of <0.05 were considered as statistically significant.

Results

General and hemodynamic characteristics of the degrees of cardiomyopathy

We observed the following changes in animals exposed to DOX (Table 1): increased heart weights ($p<0.01$ vs. sham control group) and left ventricular weights ($p<0.01$ vs. sham control group), lung congestion (as reflected by lung wet/dry weight ratio; $p<0.01$ vs. sham control group), ascites ($p<0.01$ vs. sham control group), elevated LVEDP ($p<0.01$ vs. sham control group), reduced LVSP ($p<0.01$ vs. sham control group), and decreased +dP/dt and −dP/dt ($p<0.01$ vs. sham control group).

Cardiac enzymes and proteins in blood plasma

Animal exposure to DOX led to significantly increased levels and activities of cardiac enzymes (AST, LDH, CPK, cTnI) and BNP levels in plasma ($p<0.01$ vs. sham control group); (Table 2).

Correlation of pathology score with Activity of Ca^{2+} uptake by left ventricular SR

We observed that DOX treatment significantly reduced SR Ca^{2+}

Parameter	Sham control	DOX (severe)	DOX (moderate)	DOX (mild)
n	20	15	15	10
EM score	0.14±0.05	2.46±0.16**#	1.34±0.12**##	0.66 ± 0.16**##
Body wt. (g)	414 ± 11	432 ± 17	425 ± 24	421 ± 25
Heart wt. (g)	1.25 ± 0.20	1.69 ± 0.20**	1.55 ± 0.13**#	1.41 ± 0.19**#
LV wt. (g)	0.85 ± 0.13	1.20 ± 0.14**	1.06 ± 0.10**##	1.02 ± 0.15**##
Ascites (ml)	ND	2.90 ± 0.8**	2.16 ± 0.4**##	2.01 ± 0.5**##
Lung wet/dry wt.	3.60 ± 0.3	4.52 ± 0.3**	4.31 ± 0.2**#	4.17 ± 0.2**#
Heart rate (beat/min)	201 ± 24	230 ± 27	219 ± 24	211 ± 32
LVEDP (mm Hg)	3.1 ± 1.5	8.3 ± 3.8**	6.5 ± 2.8**##	5.0 ± 2.3**##
LVSP (mm Hg)	126 ± 15	101 ± 21**	115 ± 14*#	110 ± 12*#
+dP/dt (mm Hg/s)	5020 ± 179	3920 ± 212**	4250 ± 230**##	4207 ± 220**##
−dP/dt (mm Hg/s)	5020 ± 155	3825 ± 240**	4400 ± 230**##	4250 ± 250**##

Footnote: Data are expressed as mean ± SD. DOX: doxorubicin. * p<0.05, **p<0.01 vs. sham control. #p<0.05, ##p< 0.01 vs. group DOX (Severe). LVEDP: left ventricular end diastolic pressure; LVSP: left ventricular systolic pressure; +dP/ dt: maximum rate of contraction; −dP/dt: maximum rate of relaxation

Table 1: General and hemodynamic characteristics of study groups (x ± s).

Parameters	Sham control	DOX (severe)	DOX (moderate)	DOX (mild)
n	20	15	15	10
EM score	0.14 ± 0.05	2.46 ± 0.16**	1.34 ± 0.12**##	0.66 ± 0.16**##
AST (U/L)	20.3 ± 2.4	43.7 ± 5.5**	32 ± 3.8**##	31 ± 2.9**##
LDH (U/L)	123 ± 40	351 ± 58**	254 ± 41**##	238 ± 32**##
CPK (U/L)	27.2 ± 2.1	103.2 ± 12**	80.5 ± 13**##	78.1 ± 11**##
cTnI (ng/ml)	0.2 ± 0.3	3.8 ± 0.7**	2.5 ± 0.3**##	0.9 ± 0.2**##
BNP (ng/ml)	0.30 ± 0.20	1.90 ± 0.87**	0.67 ± 0.38**##	0.82 ± 0.19**##

Footnote: Data are expressed as mean ± SD. DOX: doxorubicin. * p < 0.05, ** p < 0.01 vs. sham control. # p < 0.05, ## p < 0.01 vs. group DOX (Severe). LDH: lactate dehydrogenase; CPK: creatine kinase; AST: aspartate amino transferase; cTnI: troponin I; BNP: brain natriuretic peptide

Table 2: Biochemical parameters in study groups (x ± s).

uptake compared with sham control group (p<0.01; Table 3). The ability of SR Ca^{2+} uptake was decreased in doxorubicin-treated rats as compared with control rats and the magnitude of the decrease in SR Ca^{2+} uptake correlated with the severity of the cardiomyopathy graded by pathology score (light and electron microscopy). It was shown that the function of SR uptake and the degree of myopathy were correlated (Table 3).

Correlation of pathology score with activity of Ca^{2+}-stimulated ATPase of left ventricular SR

Animals treated with DOX exhibited significantly diminished Ca^{2+}- stimulated ATPase activity compared with sham control group (p<0.01; Table 4).

Discussion

DOX is one of the key anthracycline for the treatment of cancer patients. Effective anticancer therapy with DOX is severely limited by side effects such as cardiomyopathy and congestive heart failure [17,18]. The pathogenesis of DOX-induced cardiomyopathy has not yet been fully understood, but present studies provide a good insight into this pathology and clearly indicate the involvement of myocardial sarcoplasmic reticulum Ca^{2+} stimulated ATPase. In our investigation, we focused on studying the changes in sarcoplasmic reticulum Ca^{2+} handling, cardiac enzyme activity, and ultra structure of myocytes. The results of this study have confirmed that 12 equal cumulative doses of DOX (1.5 mg/kg, i.v.) induces chronic cardiomyopathy in rats, which is consistent with the previous studies reported by other investigators [19,20]. In this study, we demonstrate that DOX cardiomyopathy in rats is associated with clinical signs of congestive heart failure, such as appearance of ascites, lung congestion, cardiac hypertrophy and depressed left ventricular function. These changes are due to myocardial toxicity caused by DOX and are consistent with previous observations in the same experimental model of congestive heart failure [21]. Similarly, the observed decreases in the SR Ca^{2+} uptake, Ca^{2+} pump ATPase in the failing heart are due to DOX myocardial toxicity and are in line with earlier reports [9,12, 22].

Serum CPK, LDH , AST, cTn I and brain natriuretic peptide (BNP) are important myocardial enzymes in the evaluation of myocardial injury and congestive heart failure. The serum levels of these enzymes were significantly elevated in the DOX treated group.

Our studies demonstrate that the cardiomyopathy associated with chronic doxorubicin exposures is accompanied by a decrease in the amount of calcium uptake and calcium release of the sarcoplasmic reticulum as detected by a decrease in SR Ca^{2+} in uptake and by a increase in SR Ca^{2+} release. Further, the decrease in amount of calcium uptake correlates with the severity of the cardiomyopathy scored by the microscopic criteria of Billingham [5].

This study is unique in two aspects. The first is the use of pair-fed controls, since various nutritional deficiencies, such as selenium deficiency, are known to cause their own myopathic changes. We felt it was crucial to have controls that were better matched in nutritional status. Second, this study is a multilevel study correlating changes in morphology scored by light and electron microscopy, and changes in subcellular functions resulting from chronic doxorubicin exposure.

The results support the hypothesis that the sarcoplasmic reticulum is affected early in the doxorubicin cardiomyopathy as suggested by the sarcotubular dilation noted on histopathology [8,22,23]. It appears to be a specific effect on the calcium pump protein and calcium release channel of the sarcoplasmic reticulum. The decrease in amount of

Parameters	Sham control	DOX (severe)	DOX (moderate)	DOX (mild)
n	20	15	15	10
EM score	0.14 ± 0.05	2.46 ± 0.16**	1.34 ±0.12**##	0.66 ± 0.16**##
nmol Ca^{2+}/mg protein/min	164 ± 32	59 ± 11**	132 ± 25##	113 ± 23*##

Footnote: Data are expressed as mean ± SD. DOX: doxorubicin. * $p < 0.05$, ** $p < 0.01$ vs. sham control. # $p < 0.05$, ## $p < 0.01$ vs. group DOX (Severe)

Table 3: Ca^{2+} uptake by left ventricular sarcoplasmic reticulum in study groups (x ± s).

Parameters	Sham control	DOX (severe)	DOX (moderate)	DOX (mild)
n	20	15	15	10
EM score	0.14 ± 0.05	2.46 ± 0.16**	1.34 ± 0.12**##	0.66 ± 0.16**##
μmol pi / mg protein / min	0.36 ± 0.14	0.14 ± 0.05**	0.21 ± 0.12**##	0.22 ± 0.11**##

Footnote: Data are expressed as mean ± SD. DOX: doxorubicin. * $p < 0.05$, ** $p < 0.01$ vs. sham control. ## $p < 0.01$ vs. group DOX (Severe)

Table 4: Activity of sarcoplasmic reticulum Ca^{2+}stimulated ATPase in study groups (x±s).

calcium uptake resulting from chronic doxorubicin treatment model seems to be similar to that an *in vitro* doxorubicin exposure but the decrease in amount of release channel resulting from chronic doxorubicin treatment seems, at first glance, to be very different from the increased calcium sensitivity of the channel resulting from that *in vitro* doxorubicin exposure. One explanation linking these two observations would be that the decrease in calcium release channel in the chronic model, results from a down regulation of the channel in response to chronic stimulation due to increased calcium sensitivity. We did detect a small shift in calcium sensitivity in the chronic model.

Although the change seen in the calcium pump protein calcium release channel of the sarcoplasmic reticulum are not proved to be causal for the doxorubicin cardiomyopathy, the correlation with degree of myopathy and the agreement with prior morphological and *in vitro* studies suggesting the calcium pump and calcium release channel might be a target of doxorubicin.

Mechanisms for doxorubicin toxicity suggested by early studies include free radical generation and lipid peroxidation. Reactive sulfhydryl groups, being to channel regulatory sites, inhibited mRNA/protein synthesis. The results to date have been conflicting, possibly complicated by the potential of two different mechanisms, one for acute toxicity and one for the late (chronic) toxicity; or by the potential of different mechanisms during therapeutic dosing versus much higher dosing; or by different mechanisms for doxorubicin toxicity in various conditions. Furthermore, some mechanisms, such as inhibition of mRNA synthesis, might be expected to be less specific for the calcium release channel than direct interactions with the calcium release channel. Therefore, understanding the mechanism behind the apparent decrease in calcium release channel and calcium uptake protein may also help to elucidate the degree of specificity of the sarcoplasmic reticulum as an early site of injury.

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