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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 cardiomyopathy 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 Ca^{2+} loading.

Results: The ability of SR Ca^{2+} uptake was decreased in doxorubicin-treated rats compared with control rats and the magnitude of the decrease in SR Ca^{2+} 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 e ective 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]. e 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. e earliest changes are distended sarcoplasmic reticulum and early myo brillar loss. Later changes suggest di use cell damage with degeneration of multiple cellular organelles. ese early morphologic abnormalities of the sarcoplasmic reticulum have been described in animal models as Well [6]. e 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]. e 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* e ects 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 bers [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 e ects on the calcium-dependent ATPase than on the calcium release channel. us, 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 e ects *in vivo*. e purpose of this study was to assess the e ects 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. e 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. e sham control group comprised 20 animals and received standard chow diet for 6 weeks. e 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

e animals were anesthetized with intraperitoneal injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). e right carotid artery was exposed and cannulated with a microtip pressure transducer which was introduced via proximal arteriotomy. e catheter was carefully advanced through the lumen of the carotid artery until the tip of the transducer entered the le ventricle. e 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. e Le Ventricular End Diastolic Pressure (LVEDP), Le 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

A er 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

Le ventricular papillary muscles were scored by a cardiovascular pathologist in a blinded manner. e severity of doxorubicin-induced myopathic changes was graded by light microscopy and con rmed 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. e score of <1 was considered mild, 1~2 moderate, and >2 severe.

Isolation of SR membrane

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**##
n	101 q 21**	1.55 V1 0.4**##	4207 5261 0.5**##	42

uptake compared with sham control group ($p<0.01$; Table 3). e 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 le ventricular SR

Animals treated with DOX exhibited signi cantly 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. E ective anticancer therapy with DOX is severely limited by side e ects such as cardiomyopathy and congestive heart failure [17,18]. e 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. e results of this study have con rmed 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 le ventricular function. ese changes are due to myocardial toxicity caused by DOX and are consistent with previous observations for 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. e serum levels of these enzymes were signi cantly 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+} 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].

is study is unique in two aspects. e rst is the use of pair-fed controls, since various nutritional de ciencies, such as selenium de ciency, 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.

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

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 rst glance, to be very di erent 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 shi 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 sul ydryl groups, being to channel regulatory sites, inhibited mRNA/protein synthesis. e results to date have been con icting, possibly complicated by the potential of two di erent mechanisms, one for acute toxicity and one for the late (chronic) toxicity; or by the potential of di erent mechanisms during therapeutic dosing versus much higher dosing; or by di erent mechanisms for doxorubicin toxicity in various conditions. Furthermore, some mechanisms, such as inhibition of mRNA synthesis, might be expected to be less speci c for the calcium release channel than direct interactions with the calcium release channel. erefore, understanding the mechanism behind the apparent decrease in calcium release channel and calcium uptake protein may also help to elucidate the degree of speci city of the sarcoplasmic reticulum as an early site of injury.

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