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Abstract

Acute doxorubicin (DOX) cardiotoxicity can be attenuated by exercise preconditioning, but little is known of whether this cardioprotection continues beyond 10 days post-DOX administration. The purpose of this study was to determine the effects of exercise preconditioning on early chronic DOX-induced cardiotoxicity. Male rats were randomly assigned to sedentary, treadmill, or wheel running groups. Treadmill and wheel running animals participated in a progressive treadmill training protocol or voluntary wheel running, respectively, for 10 weeks. Following the intervention, animals were further randomized to receive either DOX (sedentary + DOX, treadmill + DOX, wheel running + DOX) or saline (sedentary + saline, treadmill + saline, wheel running + saline). All animals then remained sedentary for 4 weeks. A 22% reduction in fractional shortening was observed in left ventricles from previously sedentary animals receiving DOX when compared with sedentary + saline. This degree of decline was not observed in treadmill + DOX and wheel running + DOX. Sedentary + DOX possessed significantly depressed mitral and aortic valve blood flow velocities when compared with sedentary + saline, but these decrements were not observed in treadmill + DOX and wheel running + DOX. Ex vivo analysis revealed that left ventricular developed pressure and maximal rate of pressure development were significantly lower in sedentary + DOX when compared to sedentary + saline. Treadmill and wheel running prior to DOX treatment protected against these decrements. Exercise cardioprotection was associated with preserved myosin heavy chain but not sarcoendoplasmic reticulum Ca²⁺ ATPase 2a expression. In conclusion, 10 weeks of prior exercise protected against early chronic DOX cardiotoxicity suggesting that training status may be a determining factor in the degree of late-onset cardiotoxicity experienced by cancer patients undergoing treatment with DOX.

Keywords

anthracycline, cardiomyopathy, heart failure, myosin heavy chain, SERCA2a

Introduction

Cardiotoxicity associated with the anticancer drug doxorubicin (DOX, trade name Adriamycin) is typically attributed to myocardial oxidative stress as DOX undergoes redox cycling at complex I of the electron transport chain. This cardiotoxicity can present itself acutely or chronically. DOX-induced acute cardiotoxicity can be detected minutes to hours after DOX administration in the form of arrhythmias and hypotension.¹ Acute DOX cardiotoxicity may also be detected days following administration in the form of reduced left ventricular fractional shortening and developed pressure.^{2,3} Chronic cardiotoxicity, on the other hand, may set in weeks, months, or years following the completion of DOX treatment,^{4,5} and in many cases, the symptoms mimic those of dilated cardiomyopathy or congestive heart failure.^{6,7}

Although DOX treatment can lead to severe cardiotoxicity, it is one of the most effective antineoplastic agents available. As a result, numerous attempts have been made to develop interventions that minimize its side effects. Studies examining the effectiveness of these interventions, however, typically investigate only acute cardiotoxicity.^{8–11} Such interventions targeted against acute cardiotoxicity include the use of iron chelators,¹² antioxidants,^{13,14} statins,¹⁵ and amino acid supplementation.^{16,17} These acute DOX cardiotoxicity studies have limited clinical application, however, since chronic forms of DOX cardiotoxicity can

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manifest in the absence of severe acute cardiotoxicity.^{18,19} Our laboratory previously reported that 1 mg/kg DOX administered daily for 10 consecutive days (10 mg/kg cumulative) promoted a later onset (ie, weeks posttreatment) and more severe form of cardiac dysfunction than a bolus 10 mg/kg DOX dose in male rats,⁷ representing a model of “early chronic” DOX cardiotoxicity, which sets in weeks to months following cessation of treatment.⁵ Acute DOX cardiotoxicity is reversible and controllable clinically whereas chronic DOX cardiotoxicity is irreversible and has a much poorer prognosis.²⁰ Therefore, a need exists to develop interventions that provide lasting protection against chronic DOX cardiotoxicity.

DOX cardiotoxicity is multifaceted, and many traditional cardioprotective approaches typically target only one of the many possible mechanisms of its toxicity. A number of mechanisms have been proposed to explain DOX cardiotoxicity, including the generation of reactive oxygen species (ROS), apoptosis, DNA interference, and metabolic alterations (for review see Simunek et al⁵), and these mechanisms contribute to impaired systolic and diastolic cardiac function. DOX-induced systolic dysfunction has often been attributed to altered expression of cardiac myosin heavy chain (MHC) isoforms since DOX administration promotes a downregulation of α -MHC with a concurrent upregulation of β -MHC.^{3,21,22} Cardiomyocytes express both the fast ATPase activity α -MHC isoform and the slow ATPase activity β -MHC isoform, and their relative expression contributes to contractile performance as cardiomyocytes expressing primarily α -MHC have been shown to possess a 2-fold greater power output than cardiomyocytes expressing primarily β -MHC.²³ DOX-induced diastolic dysfunction, on the other hand, is often attributed to depressed cytosolic Ca^{2+} recycling.^{24,25} This impaired Ca^{2+} handling is typically a result of reduced sarcoendoplasmic reticulum Ca^{2+} ATPase 2a (SERCA2a) expression^{26,27} as this pump is responsible for the majority of Ca^{2+} recycling from the cytosol into the sarcoplasmic reticulum (SR). Because the etiology of DOX cardiotoxicity is so complex, an approach that targets multiple mechanisms could prove to be valuable clinically.

Exercise training is one such intervention that shows promise in combating multiple aspects of DOX-induced cardiotoxicity by improving antioxidant status,²⁸ attenuating apoptotic pathways,²⁹ and preserving contractile protein expression.²¹ In addition, our laboratory has reported that long-term exercise preconditioning protected against early-onset DOX cardiotoxicity up to 10 days following treatment.^{2,3} However, at this time it is unclear how long the protective effects of exercise preconditioning persist after DOX treatment is completed. The importance of this is apparent considering the fact that the clinical presentation of chronic DOX-induced cardiac dysfunction is more severe than the acute dysfunction (ie, reversible cardiac

dysfunction vs irreversible cardiac dysfunction). Therefore, the purpose of this study was to examine the lasting effects of exercise preconditioning on chronic DOX cardiotoxicity. In addition, cardiac MHC and SERCA2a expression was analyzed to investigate possible mechanisms that may explain any observed cardioprotection. It was hypothesized that exercise preconditioning would attenuate DOX-induced early chronic cardiotoxicity by attenuating MHC and SERCA2a alterations.

Materials and Methods

Animals and Animal Care

All procedures were approved by the Institutional Animal Care and Use Committee and were in compliance with the Animal Welfare Act guidelines. Male Sprague–Dawley rats (~250 g) were purchased from Harlan (Indianapolis, IN) and were housed in an environmentally controlled facility on a 12:12 light:dark cycle and were provided chow and water ad libitum. Initially, rats were randomly assigned to the sedentary group (SED, $n = 25$), treadmill training group (TM, $n = 29$), or voluntary wheel running group (WR, $n = 40$).

Exercise Preconditioning

SED animals remained in their cages throughout the 10-week treatment period. TM animals ran on a motorized rodent treadmill 5 days per week for 10 weeks using a progressive treadmill training protocol (Table 1). This treadmill protocol was shown previously to attenuate DOX-induced cardiac dysfunction 5 and 10 days following drug administration.³ WR animals were housed individually in cages fitted with voluntary running wheels and were allowed 24-hour access to the wheels for 10 weeks. Running wheel activity data were collected using a Vital View data acquisition system (MiniMitter, Bend, OR).

Doxorubicin Treatment

Twenty-four hours after the completion of the 10-week exercise preconditioning period, SED, TM, and WR animals were randomly assigned to receive either DOX or saline (SAL) injections. TM + DOX ($n = 17$), WR + DOX ($n = 23$), and SED + DOX ($n = 14$) received 1 mg/kg DOX intraperitoneally (i.p.) daily for 10 consecutive days for a cumulative dose of 10 mg/kg. Injections were performed using a stock solution of 2 mg/mL, which resulted in injection volumes of approximately 0.2 to 0.4 mL. This daily dosing scheme has been shown previously to promote later onset cardiac dysfunction (ie, early chronic cardiotoxicity) while improving survival when compared with a bolus dose.⁷ Animals in SED + SAL ($n = 11$), TM + SAL ($n =$

Table 1. Progressive Treadmill Training Protocol

	Week									
	1	2	3	4	5	6	7	8	9	10
Speed (m/min)	20	25	25	30	30	30	30	30	30	30
Incline (°)	0	0	0	3	6	9	12	15	18	18
Duration (min)	20	30	30	60	60	60	60	60	60	60

= 12), and WR + SAL (n = 17) groups received daily i.p. injections of 0.9% saline for 10 days at equivalent volumes to that of DOX. Following the 10-day DOX/SAL treatment regimen, all animals remained sedentary for 4 weeks.

Echocardiography

Four weeks following the completion of DOX treatment, cardiac function was assessed in vivo using transthoracic echocardiography using a commercially available echocardiographic system (Toshiba Nemio 30; 10 MHz pediatric transducer) as described previously by our laboratory.⁷ Briefly, the anterior and left lateral thoracic region of sedated animals (ketamine 40 mg/kg i.p.) was shaved, and the transducer was positioned to obtain left ventricular (LV) M-mode images for determination of septal wall thickness at systole (SWs) and diastole (SWd), posterior wall thickness at systole (PWs) and diastole (PWd), LV end-systolic diameter (LVDs) and LV end-diastolic diameter (LVDd). In addition, the aforementioned variables were used to calculate fractional shortening (FS), LV mass, and relative wall thickness (RWT).

Pulsed wave Doppler images were then acquired from an apical view to obtain profiles of mitral and aortic valve blood flow. Measurements taken at the mitral valve yielded maximal blood flow velocity (MV_{max}), and mean blood flow velocity (MV_{mean}). Similarly, measurements taken at the aortic valve yielded maximal blood flow velocity (AV_{max}) and mean blood flow velocity (AV_{mean}). For all echocardiography measures, data from 3 consecutive cardiac cycles, when possible, were obtained and averaged for each animal.

Isolated Working Heart

On completion of echocardiographic procedures, hearts were perfused using an isolated working heart preparation as described previously.³ Animals were anesthetized with heparinized (500 U) sodium pentobarbital (50 mg/kg). Once a tail-pinch reflex was absent, the heart was rapidly excised, the aorta was cannulated, and retrograde perfusion ensued with Krebs-Hanseleit buffer (120 mM NaCl, 5.9 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 17 mM glucose, and 0.5 mM EDTA, pH 7.4) aerated with 95% O_2 /5% CO_2 . The pulmonary vein was then

isolated and cannulated, and flow was redirected from the aorta to the left atria to initiate the working heart mode. Following stabilization, a standardized preload and afterload was obtained for each heart (10 cm H_2O and 100 cm H_2O , respectively). A micotip pressure transducer (SPR-671; 1.4 F; Millar Instruments Inc, Houston, TX) was inserted into the LV cavity for acquisition of developed pressure (LVDP), maximal rate of pressure development ($+dP/dt$), and maximal rate of pressure decline ($-dP/dt$). For all data collection, hearts were paced at 300 bpm using a stimulus isolator (ADInstruments, Colorado Springs, CO). Following the isolated working heart preparation, hearts were dissected, and the LV was isolated, snap frozen in liquid N_2 , and stored at $-80^\circ C$ for ensuing biochemical analysis.

Myosin Heavy Chain

LV homogenates were analyzed for MHC isoform expression using SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) according to Talmadge and Roy³⁰ and described previously by our laboratory.³ Frozen LVs were minced and homogenized in homogenizing buffer (250 mM sucrose, 100 mM KCl, 5 mM EDTA, and 20 mM Tris-Base, pH 6.8) and centrifuged at 1000g to isolate myofibrils. Myofibrils were then washed in washing buffer (175 mM KCl, 0.5% Triton X-100, 2 mM EDTA, and 20 mM Tris-Base, pH 6.8) twice to remove contaminating ATPases. Myofibrils were then resuspended in final resuspension buffer (150 mM KCl and 20 mM Tris-Base, pH 7.0) and protein concentration was determined using the Bradford method.³¹ Protein (9.75 μg) was loaded onto an 8% polyacrylamide separating gel with a 4% polyacrylamide stacking gel and run at 100 V (Sure-Lock electrophoresis unit, Invitrogen Corporation, Carlsbad, CA) until tracking dye reached bottom of the gel. Gels were stained with Coomassie blue, and α - and β -isoforms were analyzed using densitometry.

SERCA2a

Frozen LVs were minced and homogenized in 10 volumes of RIPA buffer containing a protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO) using a glass tissue grinder. Cell membranes were further disrupted using sonication. Homogenates were centrifuged for 10 minutes at 10 000g and the supernatant was recovered for analysis. Protein concentration was determined using the Bradford method.³¹ All samples were then normalized to 3 mg/mL protein with RIPA buffer and diluted to 1.5 mg/mL with Lamli sample buffer. 30 μg of protein (ie, 20 μL) from each sample were loaded onto lanes of 10% polyacrylamide gels and separated using SDS-PAGE. Proteins were transferred to nitrocellulose membranes and incubated with a

primary antibody specific to SERCA2a (AbCam, Cambridge, MA). The primary antibody was then probed with an alkaline phosphatase conjugated secondary antibody (WesternBreeze, Invitrogen, Carlsbad, CA) and incubated with a chromogenic substrate (BCIP/NBT) until bands developed on the membrane. Protein band intensity was analyzed using densitometry.

Statistical Analysis

All results are expressed as mean \pm standard error of the mean (mean \pm SEM). A one-way analysis of variance was performed to identify group differences between SED + SAL, SED + DOX, TM + SAL, TM + DOX, WR + SAL, and WR+DOX. If a significant *F*-value was observed, Tukey's post hoc testing was done to identify where differences existed. Additionally, WR + SAL and WR + DOX weekly wheel run distances were compared using *t* tests. For all procedures, significance was set at the $\alpha = .05$ level.

Results

General Observations

During the 10-week exercise preconditioning period, no significant weekly wheel running differences were observed between WR + SAL and WR + DOX ($P > .05$). Additionally, post hoc testing revealed that no significant differences existed between any of the variables from SED + SAL, TM + SAL, and WR + SAL ($P > .05$) suggesting that the 10-week treadmill and wheel running preconditioning treatments alone had no effect on cardiac function, MHC distribution, or SERCA2a expression when analyzed 4 weeks post-SAL administration. Therefore, significant differences reported hereafter are comparisons between SED + SAL, SED + DOX, TM + DOX, and WR + DOX, which represent the aims of this study.

Survival rates between DOX-treated groups are also worthy of note. In SED + DOX, a 60% survival rate was observed 4 weeks post-DOX administration resulting in $n = 9$ for cardiac function and biochemical analyses. In comparison, TM + DOX had a 94% survival rate and WR + DOX had a 74% survival rate resulting in $n = 16$ and $n = 17$, respectively, for cardiac function and biochemical analyses.

Echocardiography

Table 2 contains cardiac geometry data derived from M-mode images obtained during echocardiography. SED + DOX possessed significantly lower SWs and SWd when compared with SED + SAL, TM + DOX, and WR + DOX ($P < .05$) suggesting that exercise preconditioning preserved DOX-induced septal wall thinning. However, PWd was significantly lower in all DOX-treated groups when com-

pared with SED + SAL ($P < .05$) regardless of sedentary or exercise preconditioning treatments. PWd was significantly lower in SED+DOX and TM + DOX when compared with SED + SAL ($P < .05$), but this PWd decrement was not observed in WR + DOX ($P > .05$). No between-group differences were observed in LVDs or LVDd ($P > .05$), but RWT and LV mass were found to be significantly lower in SED + DOX when compared with SED + SAL, TM + DOX, and WR + DOX ($P < .05$). A 21% decrease in FS was observed in SED + DOX when compared with SED + SAL, which was found to be significant (Figure 1, $P < .05$). Although TM + DOX and WR + DOX possessed a 13% and 15% decrease in FS, respectively, when compared with SED + SAL, these differences were not found to be significant ($P > .05$).

Mitral valve blood flow velocities are illustrated in Figure 2. When compared with SED + SAL, SED + DOX possessed a 37% reduction in MV_{max} ($P < .05$, Figure 2A), and although TM + DOX and WR + DOX had 12% and 14% reductions in MV_{max} , respectively, when compared with SED+SAL, these differences were not different from SED + SAL ($P > .05$). Furthermore, SED + DOX MV_{max} was found to be significantly lower than TM + DOX and WR + DOX ($P < .05$). MV_{mean} was significantly lower in SED + DOX and WR + DOX when compared with SED + SAL (-38% and 17%, respectively, $P < .05$, Figure 2B), but TM + DOX MV_{mean} was not found to differ significantly from SED + SAL (-12%, $P > .05$). Additionally, TM + DOX and WR + DOX MV_{mean} was significantly greater than SED + DOX ($P < .05$).

Blood flow measurements taken at the aortic valve are illustrated in Figure 3. AV_{max} was significantly lower in SED + DOX when compared with SED + SAL (-47%, $P < .05$, Figure 3A). However, TM + DOX and WR + DOX was not significantly different than SED + SAL AV_{max} (-4% and -9%, respectively, $P > .05$), but TM + DOX and WR+DOX AV_{max} was significantly greater than SED + DOX ($P < .05$). AV_{mean} was significantly lower in SED + DOX when compared with SED + SAL (-48%, $P < .05$, Figure 3B), but this difference was not seen in TM + DOX and WR + DOX (-5% and -14%, respectively, $P > .05$). TM + DOX and WR + DOX AV_{mean} were significantly greater than SED + DOX ($P < .05$).

It is also important to note that in vivo heart rates derived from Doppler imaging were found to be significantly different between groups. SED + DOX heart rate was found to be lower than SED + SAL (302 ± 14 and 390 ± 8 bpm, respectively, $P < .05$). Exercise preconditioning, however, attenuated DOX-induced heart rate reductions as this difference was not observed in TM + DOX and WR + DOX (356 ± 10 and 343 ± 13 bpm, respectively, $P > .05$).

Isolated Working Heart

Using the isolated working heart model allows for standardized loading of each heart (ie, preload, afterload, heart

Table 2. M-Mode Derived Cardiac Geometry^a

	SED + SAL	SED + DOX	TM + SAL	TM + DOX	WR + SAL	WR + DOX
SWs (mm)	3.06 ± 0.05	2.24 ± 0.13 ^{b,c,d}	3.11 ± 0.12	2.81 ± 0.09	2.89 ± 0.09	2.73 ± 0.07
SWd (mm)	1.74 ± 0.06	1.28 ± 0.11 ^{b,c,d}	1.81 ± 0.07	1.73 ± 0.06	1.75 ± 0.06	1.70 ± 0.05
PWs (mm)	3.32 ± 0.08	2.35 ± 0.14 ^b	3.30 ± 0.14	2.61 ± 0.07 ^b	3.18 ± 0.14	2.68 ± 0.08 ^b
PWd (mm)	1.86 ± 0.06	1.26 ± 0.13 ^{b,d}	1.96 ± 0.08	1.54 ± 0.04 ^b	1.82 ± 0.08	1.68 ± 0.07
LVDs (mm)	3.17 ± 0.17	3.94 ± 0.11	3.37 ± 0.21	3.57 ± 0.17	3.06 ± 0.21	3.68 ± 0.14
LVDd (mm)	6.51 ± 0.15	6.63 ± 0.14	6.75 ± 0.16	6.49 ± 0.27	6.49 ± 0.17	6.52 ± 0.10
RWT (mm)	0.55 ± 0.02	0.39 ± 0.03 ^{b,c,d}	0.58 ± 0.02	0.55 ± 0.03	0.56 ± 0.03	0.52 ± 0.02
LV mass (mg)	788 ± 36	459 ± 70 ^{b,c,d}	905 ± 47	745 ± 43	777 ± 31	720 ± 25

Note: SED = sedentary; SAL = saline; DOX = doxorubicin; TM = treadmill; WR = wheel running; SWs = septal wall thickness at systole; SWd = septal wall thickness at diastole; PWs = posterior wall thickness at systole; PWd = posterior wall thickness at diastole; LVDs = left ventricular dimension at systole; LVDd = left ventricular dimension at diastole; RWT = relative wall thickness; LV = left ventricle.

^aData are mean ± standard error of the mean.

^b $P < .05$ versus SED + SAL.

^c $P < .05$ versus TM + DOX.

^d $P < .05$ versus WR + DOX.

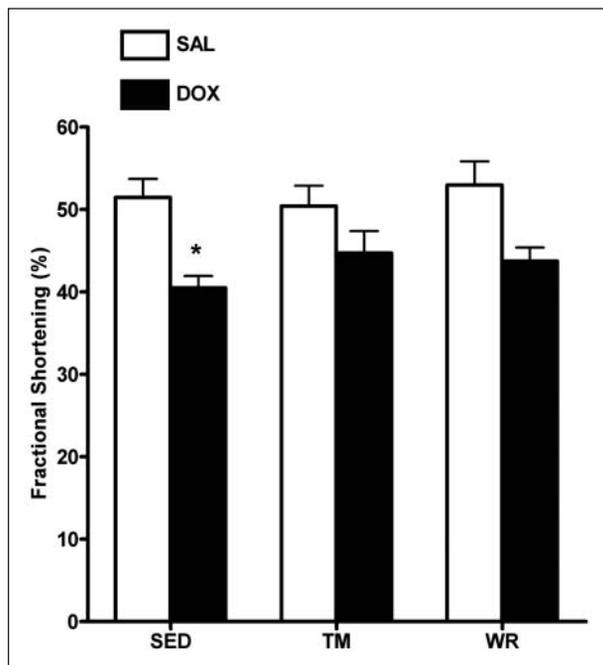


Figure 1. Left ventricular fractional shortening of sedentary (SED), treadmill trained (TM), and wheel run (WR) rats receiving doxorubicin (DOX) or saline (SAL)

* $P < .05$ versus SED + SAL.

rate), and it was observed that SED + DOX possessed significantly lower LVDP than SED + SAL, TM + DOX, and WR + DOX (-22% , -16% , and -19% , respectively, $P < .05$, Figure 4A). Maximal rate of pressure development ($+dP/dt$) was significantly lower in SED + DOX when compared with SED + SAL and WR + DOX (-19% and -15% , respectively, $P < .05$, Figure 4B), and no significant difference was observed

between TM + DOX and SED + DOX ($P > .05$). No significant group differences were observed with $-dP/dt$ ($P > .05$, Figure 4C).

Myosin Heavy Chain and SERCA2a expression

Cardiac MHC is expressed in 2 isoforms (α and β), and various forms of cardiac dysfunction (including DOX-induced cardiotoxicity) are associated with upregulation of the β -isoform.^{3,23} In the present study, LVs from SED + DOX expressed 26% β -MHC (with corresponding 74% α -MHC), which was significantly different than the 17% β -MHC (with corresponding 83% α -MHC) expressed in LVs from SED + SAL ($P < .05$, Figure 5). TM + DOX and WR + DOX LVs, however, did not express a significantly different MHC isoform expression when compared with SED + SAL (21% β -MHC and 21% β -MHC, respectively, $P > .05$). Although treadmill and wheel running provided protection against DOX-induced MHC alterations, exercise preconditioning did not provide protection against DOX-induced SERCA2a alterations. LVs from SED + DOX, TM + DOX, and WR + DOX expressed significantly lower levels of SERCA2a than SED + SAL ($P < .05$, Figure 6).

Discussion

The present study reports the protective effects of exercise preconditioning on chronic DOX-induced cardiotoxicity. Overall, exercise preconditioning (10 weeks of treadmill training and wheel running) attenuated DOX-induced cardiac dysfunction analyzed 4 weeks post-DOX treatment. This cardioprotection was associated with preserved β -MHC expression, but exercise preconditioning did not protect against DOX-induced SERCA2a downregulation.

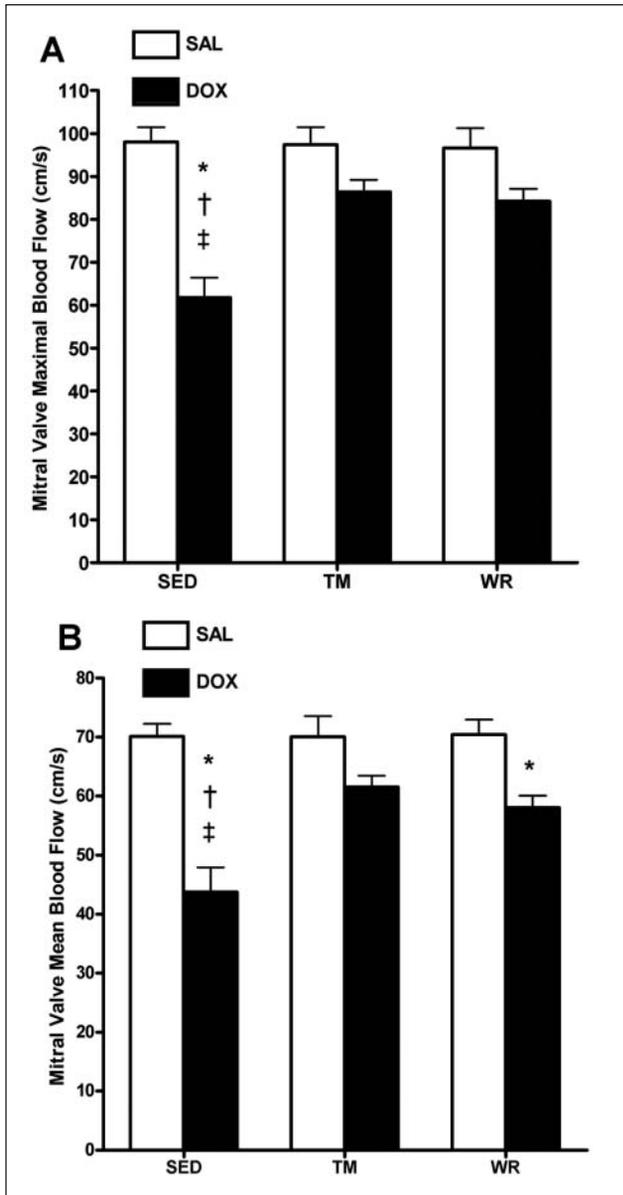


Figure 2. Mitral valve blood inflow velocities of sedentary (SED), treadmill trained (TM), and wheel run (WR) rats receiving doxorubicin (DOX) or saline (SAL)
A, Maximal blood flow velocity. B, Mean blood flow velocity. * $P < .05$ versus SED + SAL; † $P < .05$ versus TM + DOX; ‡ $P < .05$ versus WR + DOX.

Doxorubicin Cardiotoxicity

DOX cardiotoxicity is associated with the formation of ROS (oxidative stress), which in turn can affect MHC³² and SERCA.³³ The rat heart expresses primarily the fast ATPase activity α -MHC isoform with minimal expression of the slow ATPase activity β -MHC isoform. Shifting of the relative isoform expression toward an increase in β -MHC (with a corresponding decrease in α -MHC expression) may occur during congestive heart failure,³⁴ dilated cardiomyopathy,³⁵ diabetic cardiomyopathy,³⁶ and DOX

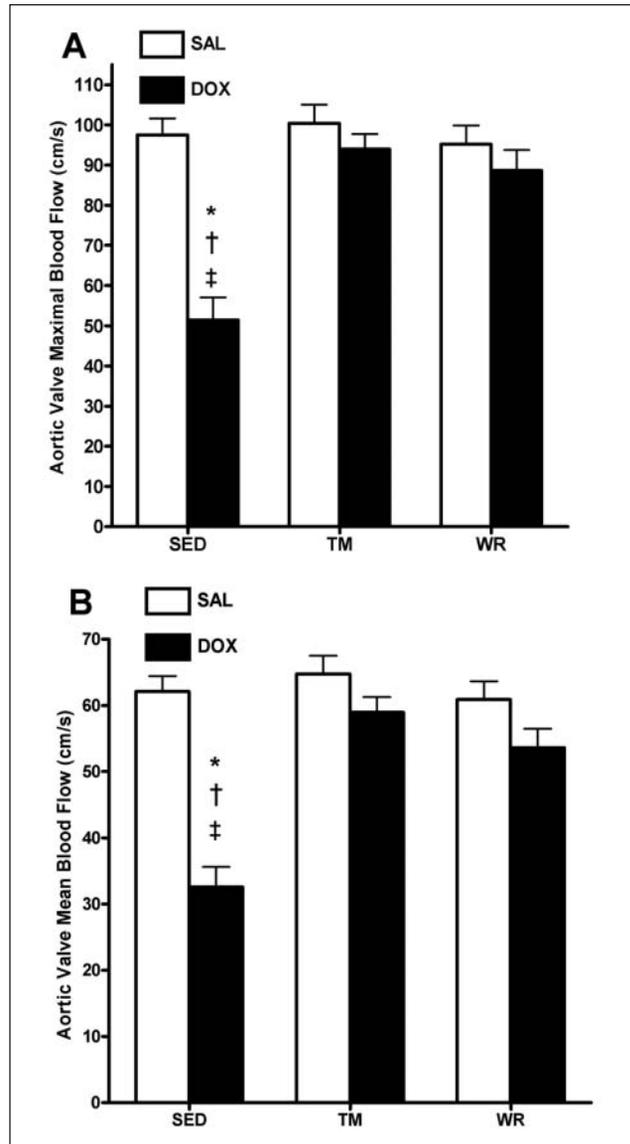


Figure 3. Aortic valve blood outflow velocities of sedentary (SED), treadmill trained (TM), and wheel run (WR) rats receiving doxorubicin (DOX) or saline (SAL)
A, Maximal blood flow velocity. B, Mean blood flow velocity. * $P < .05$ versus SED + SAL; † $P < .05$ versus TM + DOX; ‡ $P < .05$ versus WR + DOX.

exposure.^{3,21,22} This MHC shift is associated with significant impairments in cardiocyte sliding filament velocities³⁷ and power outputs^{38,39} as well as LV pressure development.⁴⁰ In addition to ROS-related MHC disturbances, DOX impairs myocardial energetics by accumulating in and disrupting mitochondria.⁴¹ The result is impaired mitochondrial function and altered ATP production, which has been shown to promote a shift in the cardiac MHC profile.⁴² A shift toward the β -isoform results in a more metabolically efficient cardiocyte; however, this energy preservation comes at the cost of systolic function.

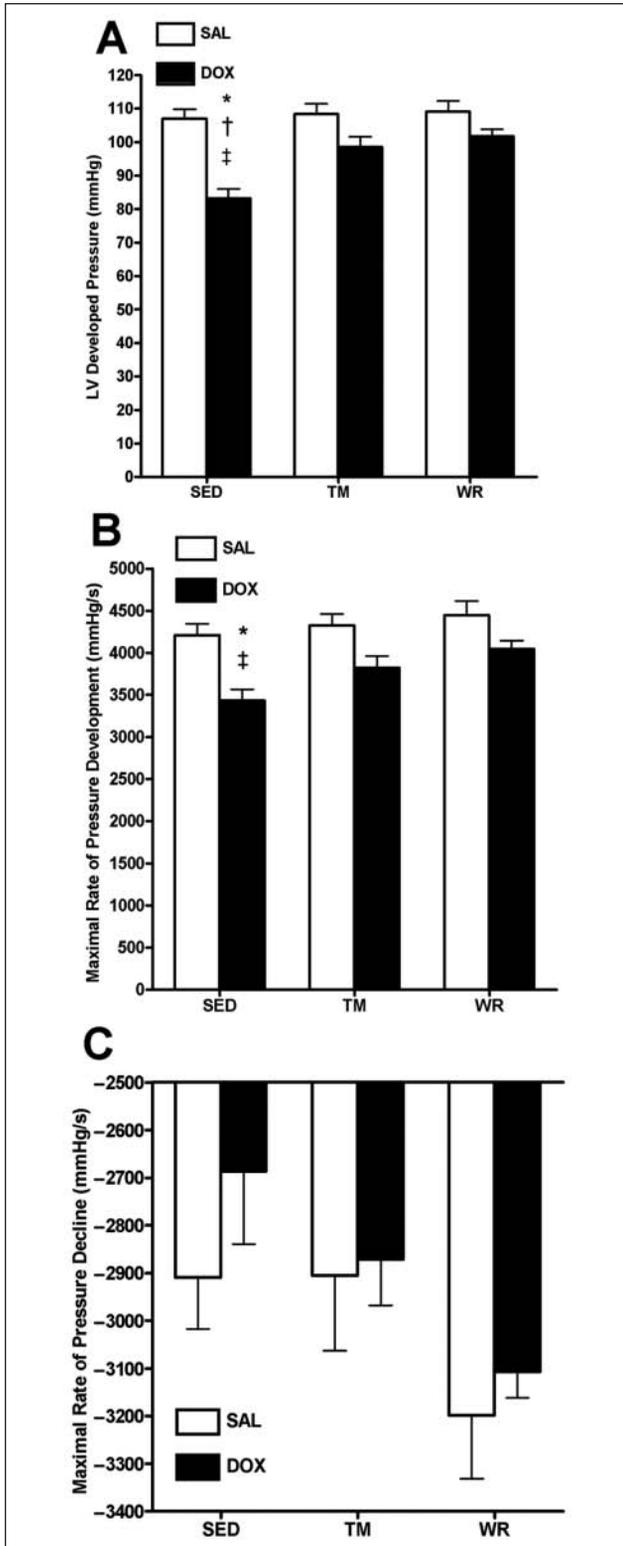


Figure 4. Isolated working heart left ventricular (LV) function of sedentary (SED), treadmill trained (TM), and wheel run (WR) rats receiving doxorubicin (DOX) or saline (SAL). A, Developed pressure. B, Maximal rate of pressure development (+dP/dt). C, Maximal rate of pressure decline (-dP/dt). * $P < .05$ versus SED + SAL; † $P < .05$ versus TM + DOX; ‡ $P < .05$ versus WR + DOX.

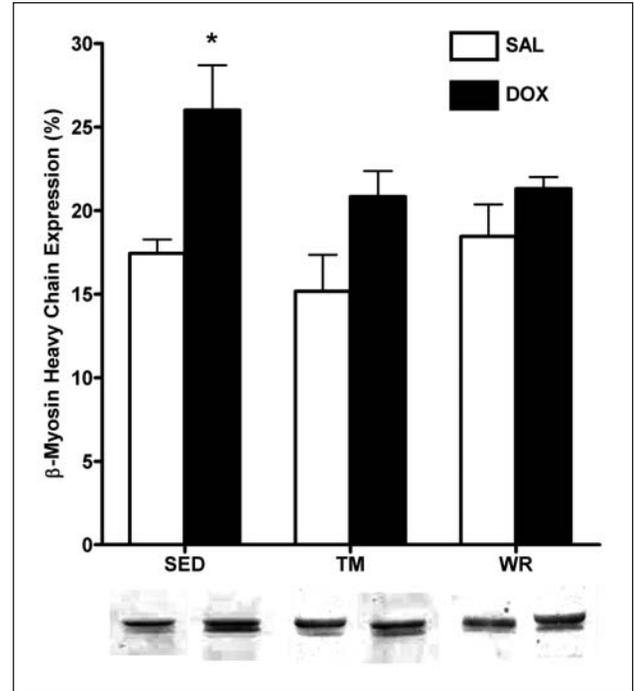


Figure 5. β -Myosin heavy chain expression, with representative gel scans, in left ventricular homogenates from sedentary (SED), treadmill trained (TM), and wheel run (WR) rats receiving doxorubicin (DOX) or saline (SAL). The upper band on the gel scan is the α -isoform and the bottom band is the β -isoform. * $P < .05$ versus SED + SAL.

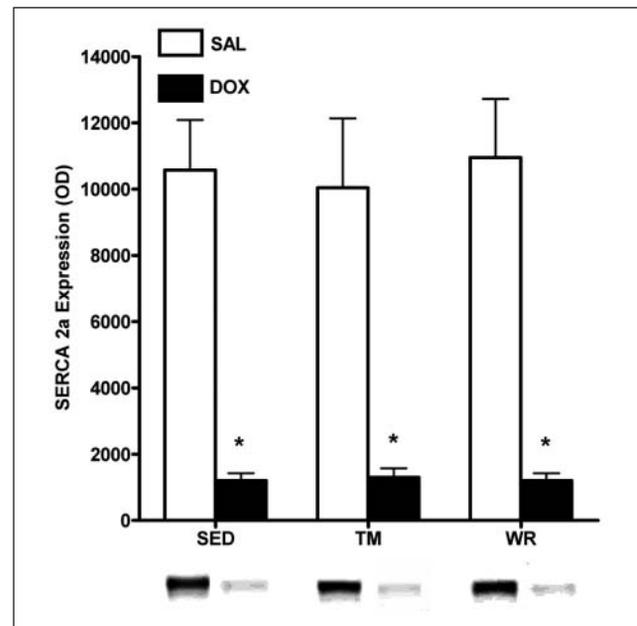


Figure 6. Sarcoendoplasmic reticulum calcium ATPase 2a (SERCA2a) expression, with representative blots, in left ventricular homogenates from sedentary (SED), treadmill trained (TM), and wheel run (WR) rats receiving doxorubicin (DOX) or saline (SAL). * $P < .05$ versus SED + SAL.

In the present study, DOX promoted β -MHC upregulation with concomitant α -MHC downregulation, and this altered MHC profile was associated with systolic dysfunction in the form of reduced aortic blood flow velocities, LVDP, and $+dP/dt$. Systolic dysfunction with DOX treatment is a common clinical concern as ejection fraction is often times measured throughout treatment to assess the degree of cardiotoxicity.¹⁸ However, the issue of chronic cardiotoxicity is equally concerning because debilitating cardiac dysfunction may set in months or years following treatment even though severe cardiac dysfunction was not detected during treatment.⁴³ Long-term cardiac remodeling as a result of the DOX insult may be more of a concern than the acute changes occurring immediately following DOX administration because of the fact that it may be clinically diagnosed as dilated cardiomyopathy or heart failure.

Left ventricular morphology in the current study was also significantly altered 4 weeks following DOX treatment. Reductions in LV wall thickness, mass, and FS were evident, and taken together, these changes suggest myocardial wasting which could be explained, at least partly, by apoptosis. Apoptosis is one of the many mechanisms contributing to DOX cardiotoxicity,⁴⁴⁻⁴⁶ and although indices of apoptosis were not analyzed in the present study, it is possible that apoptotic pathways were activated, which may have led to programmed cell death since DOX administration can increase both intrinsic and extrinsic apoptotic pathway activities.⁴⁷ Cell death, in this regard, may have contributed to the reduced wall thickness and LV mass observed with DOX-treated hearts.

DOX cardiotoxicity has also been attributed to cardiac Ca^{2+} homeostasis interruptions,^{48,49} and it has been reported that DOX treatment downregulates expression of key Ca^{2+} handling proteins such as ryanodine receptor, calsequestrin, Na/Ca exchanger, phospholamban, SERCA2a, and SERCA2b.^{26,50} In the current study, we analyzed SERCA2a expression as it is the primary SERCA isoform expressed in cardiac cells and therefore responsible for the majority of sarcoplasmic reticulum (SR) Ca^{2+} reuptake. Efficient pumping of Ca^{2+} from the cytosol to the SR is critical in cardiocyte relaxation, and one hallmark of DOX cardiotoxicity is diastolic dysfunction.^{51,52} The current study reports DOX-induced diastolic dysfunction in the form of reduced mitral valve blood flow velocities with associated downregulation of SERCA2a.

Exercise Cardioprotection

Exercise preconditioning has been shown previously to attenuate early-onset DOX cardiotoxicity.^{2,3,53,54} However, in these studies, cardiotoxicity was analyzed 1 to 10 days post-DOX administration, and until the current study, it was unclear how long the protective effects of exercise preconditioning against DOX cardiotoxicity could persist. Exercise preconditioning in the current

study protected against DOX-induced SW thinning, LV mass reduction, FS decline, mitral and aortic blood flow decrements, LVDP reduction, and $+dP/dt$ decline.

Since DOX cardiotoxicity involves the generation of ROS, many studies examining the cardioprotective effects of exercise have focused on oxidative stress and antioxidants. DOX-induced oxidative stress has been shown to cause cellular damage in the form of lipid peroxidation and protein carbonylation both of which are ameliorated with exercise preconditioning.^{53,54} Kanter et al²⁸ reported that exercise training prior to and during DOX treatment was associated with increased antioxidant activities (catalase, glutathione peroxidase, and superoxide dismutase) in blood and liver of mice, but this degree of antioxidant upregulation was not observed in the heart. Likewise, exercise-induced cardioprotection has been shown previously to not necessarily be the result of antioxidant upregulation.^{2,54} Although cardiac antioxidant upregulation was not evident in these exercise training studies at the time of myocardial analysis (ie, 1 and 5 days post DOX), it is possible that antioxidant activity/expression was increased at the time of DOX administration, which potentially provided protection against the initial insult of DOX.

One of the key findings of the current study was that exercise-induced preservation of the MHC isoform distribution was evident 4 weeks after completing DOX treatment even though the animals remained sedentary during the 4-week post-DOX period. The preservation of LV MHC isoform distribution 4 weeks post-DOX in exercise preconditioned rats is similar to the degree of preservation observed in an earlier report in which the MHC isoform distribution was measured 5 and 10 days post-DOX treatment.³ This preservation of MHC expression may explain the attenuated systolic dysfunction observed in exercise preconditioned rats since relative α - and β -MHC expression has a dramatic impact on cardiac function.^{38,39} Exercise training has a profound effect on MHC expression as illustrated by Jin et al,⁵⁵ who reported that 13 weeks of progressive exercise training substantially increased α -MHC mRNA. In fact, of the genes analyzed in the study by Jin et al,⁵⁵ α -MHC was the only one found to be significantly increased. It is possible that a training-induced upregulation of α -MHC mRNA in the current study provided protection against the initial insult of DOX, which typically promotes a decrease in α -MHC expression. This is supported by evidence that significantly altered MHC expression has been detected just hours after DOX administration.⁵⁶ The duration of the cardioprotective effects of exercise observed in the present study may in fact be related to the preconditioned heart being resistant to the acute DOX exposure thereby minimizing the chronic cardiotoxicity. This concept has been illustrated clinically by studies indicating that the severity of DOX-induced early-onset cardiac dysfunction has been used to predict the severity of chronic cardiac dysfunction.⁴³

The lack of SERCA2a preservation in exercise preconditioned hearts in the current study is also worthy of note. Despite significantly lower SERCA2a expression when compared with sedentary controls, hearts from preconditioned animals receiving DOX had significantly better diastolic function than hearts from sedentary animals receiving DOX (ie, maximal and mean mitral blood flow velocities). Although SERCA2a is primarily responsible for the Ca^{2+} reuptake into the SR, which is involved in cardiocyte relaxation, it is possible that other mechanisms may have been responsible for the preserved diastolic function observed in hearts from exercise preconditioned DOX-treated animals. One major mechanism contributing to DOX-induced cardiac dysfunction is cytosolic Ca^{2+} overload, and downregulation of SERCA2a contributes to Ca^{2+} overload-induced dysfunction.⁵⁷ However, reduced SERCA2a expression in models of cardiac dysfunction may be a compensatory mechanism,⁵⁸ and SERCA2a deficiency is not necessarily associated with reduced cardiac performance.⁵⁹ Additionally, DOX has been shown to alter other Ca^{2+} handling proteins such as ryanodine receptor, calsequestrin, and Na/Ca exchanger resulting in altered Ca^{2+} handling, and this disruption contributes to DOX cardiotoxicity.^{26,50} Exercise has been shown to have a positive impact on overall cellular Ca^{2+} translocation,^{60,61} and future investigations should focus on the interaction of DOX and exercise on additional Ca^{2+} translocation mechanisms.

Conclusion

These data demonstrate that 10 weeks of exercise preconditioning was cardioprotective against DOX cardiotoxicity. Interestingly, this cardioprotection was observed 4 weeks after the conclusion of DOX treatment even though the animals were sedentary during the 4-week period. Cardioprotection was associated with preserved in vivo and ex vivo cardiac function, preserved MHC isoform distribution, and was evident regardless of mode of activity (treadmill/wheel running). This suggests that exercise stress level was not a major factor in the degree of protection as voluntary wheel running has been shown to be less stressful than motorized treadmill running.⁶² This study provides further insight into the effects of exercise and DOX cardiotoxicity by demonstrating that exercise preconditioning can protect against DOX-induced cardiac dysfunction for up to 4 weeks after exposure (early chronic cardiotoxicity), and that exercise during the post-DOX period is not a requisite to maintain the cardioprotective effects.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

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