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ORIGINAL RESEARCH

Remote Ischemic Pre-Conditioning Attenuates Adverse Cardiac Remodeling and Mortality Following Doxorubicin Administration in Mice

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ABSTRACT

OBJECTIVES Because of its multifaceted cardioprotective effects, remote ischemic pre-conditioning (RIPC) was examined as a strategy to attenuate doxorubicin (DOX) cardiotoxicity.

BACKGROUND The use of DOX is limited by dose-dependent cardiotoxicity and heart failure. Oxidative stress, mitochondrial dysfunction, inflammation, and autophagy modulation have been proposed as mediators of DOX cardiotoxicity.

METHODS After baseline echocardiography, adult male CD1 mice were randomized to either sham or RIPC protocol (3 cycles of 5 min femoral artery occlusion followed by 5 min reperfusion) 1 h before receiving DOX (20 mg/kg, intraperitoneal). The mice were observed primarily for survival over 85 days (86 mice). An additional cohort of 50 mice was randomized to either sham or RIPC 1 h before DOX treatment and was followed for 25 days, at which time cardiac fibrosis, apoptosis, and mitochondrial oxidative phosphorylation were assessed, as well as the expression profiles of apoptosis and autophagy markers.

RESULTS Survival was significantly improved in the RIPC cohort compared with the sham cohort ($p = 0.007$). DOX-induced cardiac fibrosis and apoptosis were significantly attenuated with RIPC compared with sham ($p < 0.05$ and $p < 0.001$, respectively). Although no mitochondrial dysfunction was detected at 25 days, there was a significant increase in autophagy markers with DOX that was attenuated with RIPC. Moreover, DOX caused a 49% decline in cardiac BCL2/BAX expression, which was restored with RIPC ($p < 0.05$ vs. DOX). DOX also resulted in a 17% reduction in left ventricular mass at 25 days, which was prevented with RIPC ($p < 0.01$), despite the lack of significant changes in left ventricular ejection fraction.

CONCLUSIONS Our preclinical results suggested that RIPC before DOX administration might be a promising approach for attenuating DOX cardiotoxicity. (J Am Coll Cardiol CardioOnc 2019;1:221–34) © 2019 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

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ABBREVIATIONS AND ACRONYMS

ADP = adenosine phosphate

DOX = doxorubicin

LV = left ventricular

LVEF = left ventricular ejection fraction

RIC = remote ischemic conditioning

RIPC = remote ischemic pre-conditioning

ROS = reactive oxygen species

STEMI = ST- segment elevation myocardial infarction

The use of doxorubicin (DOX), a chemotherapeutic antibiotic from the anthracycline family, has been limited by its dose-dependent cardiotoxicity and related heart failure in cancer survivors (1,2). Immense research effort has been focused on attenuating DOX cardiotoxicity by modulating its cumulative dose or considering alternative chemotherapies; however, DOX remains among the most efficacious agents for a number of cancers, including solid and hematological malignancies. Despite careful adjustment of the cumulative dose, exposure to low or moderate doses of DOX have been associated with early evidence of subclinical cardiovascular disease (3). Studies have indicated the involvement of topoisomerase IIβ inhibition, oxidative stress, mitochondrial dysfunction, inflammation, and autophagy modulation, among several other factors, for mediating DOX-induced cardiotoxicity and subsequent cardiomyopathy (4–8). However, to date, there are no universally prescribed or widely used preventive or therapeutic modalities that mitigate DOX cardiotoxicity. Recent statistics based on 3 Phase III clinical trials estimate that 26% of cancer patients develop cardiotoxicity after exposure to a cumulative dose of 550 mg/m^2 (9), and >50% of all older adult patients with lymphoma and survivors of childhood cancer who received DOX treatment remain at high risk of developing cardiotoxicity (10).

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Ischemic pre-conditioning refers to sublethal episodes of ischemia that prepare an organ or vascular territory for subsequent prolonged ischemic events (11,12). Early animal models showed that brief transient occlusions of a coronary artery could dramatically limit myocardial infarct size following longer subsequent ischemia (13). Similar efficacy can be attained when the pre-conditioning event occurs in a remote vascular bed, a phenomenon known as remote ischemic pre-conditioning (RIPC) (14–17). The mechanism by which RIPC (or any ischemic pre-conditioning) works is not entirely clear and is almost certainly multifactorial (18).

The purpose of our study was to determine whether RIPC can limit DOX-induced cardiotoxicity in a mouse model. We evaluated the impact of RIPC applied in a clinically feasible time of 1 h before DOX administration on survival, cardiac function, left ventricular (LV) mass, cardiac fibrosis, apoptosis, and autophagy.

METHODS

ANIMALS. Adult male CD1 mice were purchased from Charles River Laboratories International, Inc. (Wilmington, Massachusetts). The animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. All animal experiments were conducted under the guidelines on humane use and care of laboratory animals for biomedical research published by the U.S. National Institutes of Health (National Institutes of Health Publication No. 85-23, revised 1996).

RIPC AND DOX. Animals were randomized to either sham control or RIPC protocol (**Figure 1**). A subgroup of age-matched naive male CD1 mice (n = 12) that did not receive RIPC or DOX treatment was used as negative control. Under anesthesia (pentobarbital 30 mg/kg intraperitoneal), all animals had their right femoral artery exposed, and a silk suture was passed around the artery. For RIPC, the femoral artery was then occluded by tightening the suture. The artery was occluded for 5 min, then the suture was relaxed for 5 min, then repeated, for a total of 3 cycles of occlusion and/or reperfusion. The control animals did not have the suture tightened. One hour after RIPC or control, all animals received a single intraperitoneal injection of DOX 20 mg/kg.

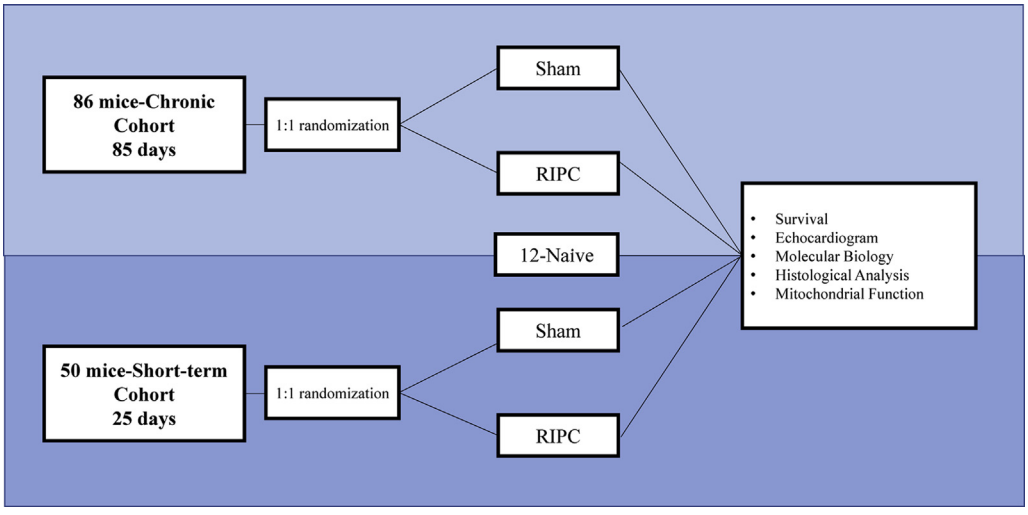
All procedures and data analysis were conducted in a blinded fashion for the various parameters.

SURVIVAL. Two cohorts were included in this study: 1) a long-term cohort that was monitored for survival for 85 days following the experimental procedure (RIPC or sham surgery) and DOX treatment; and 2) a short-term cohort that was killed after 25 days, for histological and molecular testing. There were 86 mice in the long-term cohort, randomized in 1:1 fashion to RIPC or sham control. The short-term cohort included 50 mice, similarly randomized. Survival rate was determined based on the animals that survived the experimental protocol starting at recovery following RIPC or sham surgery.

ECHOCARDIOGRAPHY. Echocardiography was performed using the Vevo2100 ultrasound system (VisualSonics, Toronto, Ontario, Canada) as previously reported (19). For methodology details, please refer to Supplemental Appendix (20,21).

ASSESSMENT OF LV FIBROSIS. Paraffin-embedded cardiac tissue sections were stained with picrosirius red to determine interstitial fibrosis as previously reported (22).

FIGURE 1 **Animal Randomization Diagram**



Consort-style diagram showing animal randomization (1:1) in both sham control (doxorubicin [DOX] alone) and remote ischemic preconditioning (RIPC)+DOX treatment groups. One hour after RIPC or sham control, all animals received a single intraperitoneal injection of DOX 20 mg/kg. Naive animals did not receive DOX or RIPC. **(Top)** Chronic cohort (85 days; 86 mice) randomization. **(Bottom)** Short-term cohort (25 days; 50 mice) randomization.

ASSESSMENT OF CARDIAC APOPTOSIS. Cardiac apoptosis was detected using the ApoAlert DNA Fragmentation Assay Kit (Clontech, Mountain View, California) that detects nuclear DNA fragmentation. A detailed description can be found in Supplemental Appendix.

MITOCHONDRIA. Mitochondria were isolated from adult mouse hearts as previously reported (23,24). A detailed description can be found in the Supplemental Appendix.

WESTERN BLOTTING. Cardiac protein extraction was performed as previously reported (25). Specific details can be found in the Supplemental Appendix.

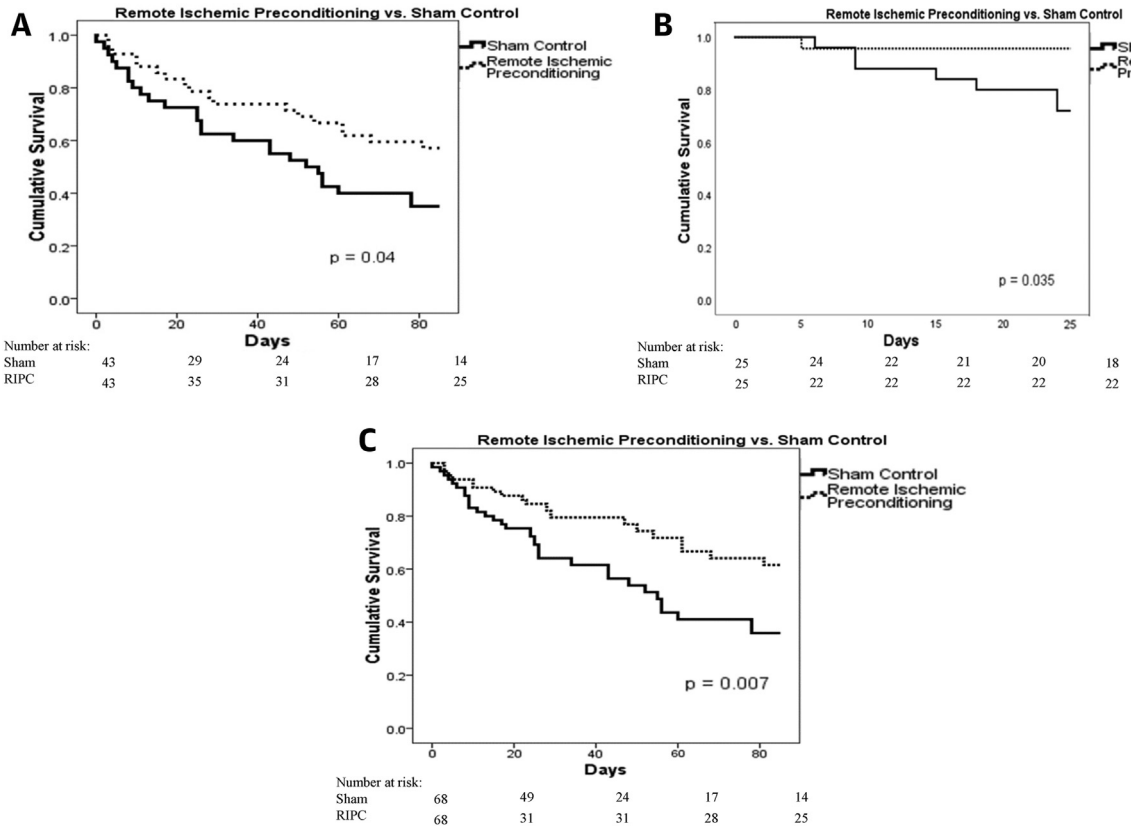
STATISTICAL ANALYSIS. Continuous variables are presented as mean ± SD for normally distributed variables and median (25th to 75th percentiles) for non-normal variables. Comparisons were made using a Student's *t*-test or Mann-Whitney *U* test for normal and non-normal variables as appropriate. Survival rates are presented by Kaplan-Meier plots, which were compared using the log-rank test. All tests of significance were 2-sided, and $p < 0.05$ was considered statistically significant. Data were analyzed using SPSS version 24 (IBM, Armonk, New York).

RESULTS

RIPC IMPROVES SURVIVAL AFTER DOX TREATMENT. Of the initial 86 mice allocated to the long-term survival cohort (85 days), 4 did not survive the RIPC or sham procedures and were excluded. The remaining 82 mice (42 RIPC and 40 sham control mice) were included in the long-term survival cohort and received DOX 1 h following RIPC or sham procedure. Survival was significantly improved in mice subjected to RIPC ($p = 0.04$) (**Figure 2A**). Of the initial 50 mice allocated to the short-term study cohort (25 days), 2 did not survive the RIPC or sham procedures and were excluded. The remaining 48 mice (23 RIPC and 25 sham control mice) made up the short-term study cohort. Similar to the long-term cohort, survival was significantly improved with RIPC compared with DOX alone at 25 days after treatment ($p = 0.035$) (**Figure 2B**). When the 2 cohorts were combined, the survival benefit of RIPC was more pronounced ($p = 0.007$) (**Figure 2C**).

RIPC PREVENTS DOX-INDUCED DECLINE IN LV MASS. Echocardiographic assessments were conducted in both short- and long-term cohorts. Baseline left ventricular ejection fraction (LVEF) was similar between the 2 groups ($p = 0.90$), did not change at 25 days following DOX treatment ($p = 0.83$) (**Figure 3A**), and remained consistent throughout the study up to 85 days in the long-term cohort. However, LV mass declined by 17% in the DOX group, which was completely blunted in the RIPC+DOX mice at 25 days following DOX treatment (**Figure 3B**). The long-axis view of global longitudinal strain rate

FIGURE 2 Survival After DOX in Mice With and Without RIPC



Kaplan-Meier curves show survival in DOX-injected mice with RIPC or sham control. **(A)** Mice in the long-term survival cohort, **(B)** mice in the short-term cohort, and **(C)** includes both long-term and short-term cohorts. Abbreviations in Figure 1.

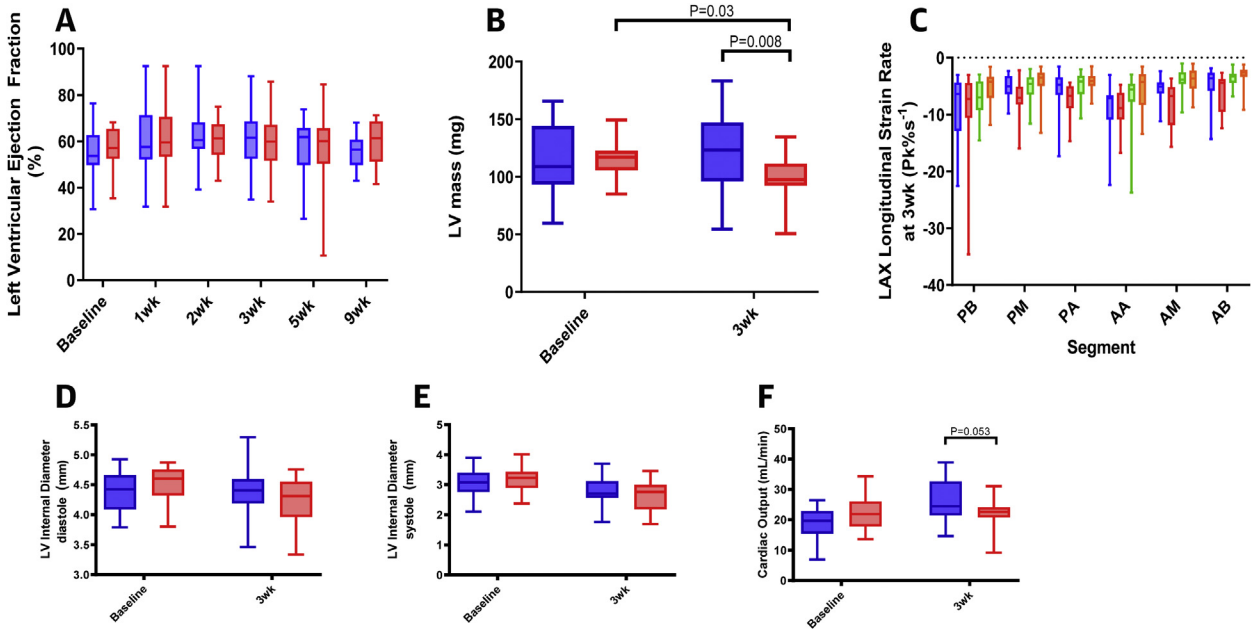
analysis did not show any difference between groups (Figure 3C). LV dimensions in diastole or systole were not significantly different at 25 days (Figures 3D and 3E). Cardiac output was significantly higher in RIPC+DOX group compared with the DOX-alone group at 25 days ($p = 0.05$) (Figure 3F). Additional cardiac parameter data are presented in Table 1.

RIPC ATTENUATES DOX-INDUCED CARDIAC FIBROSIS AND APOPTOSIS. DOX treatment resulted in significant cardiac fibrosis 25 days after administration, which was attenuated in mice subjected to RIPC ($p < 0.05$) (Figure 4). As expected, in age-matched naive mice, there was no remarkable cardiac fibrosis.

Terminal deoxynucleotidyl transferase dUTP nick end labeling staining was used to assess cardiac apoptosis in the different groups. DOX significantly increased the frequency of apoptotic nuclei compared with the naive group, which was mitigated with RIPC ($p < 0.001$) (Figure 5).

DOX OR RIPC+DOX DID NOT ALTER MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION. The rate of oxidative phosphorylation was determined in mitochondria isolated from the hearts of mice from the different groups. RIPC before DOX treatment did not alter the protein yield of mitochondria (Table 2). In addition, there were no differences in the rate of oxidative phosphorylation between the DOX group and the RIPC+DOX group using complex I or complex II substrates. The rates of adenosine phosphate (ADP)–stimulated (state 3 respiration), ADP-limited (state 4 respiration), maximal ADP-stimulated state 3 respiration (2 mM ADP), and uncoupled respiration (dinitrophenol stimulated respiration) were all similar. These similar rates of uncoupled respiration confirmed that the electron transport chain was unaltered and also excluded a functionally significant defect in the phosphorylation apparatus. The coupling of respiration as shown by the respiratory control ratio was also not affected by RIPC.

FIGURE 3 Echocardiographic Parameters 25 Days Post-DOX Treatment With or Without RIPC



(A) Despite no change in left ventricular (LV) ejection fraction at 25 days following DOX treatment in either group, **(B)** LV mass significantly declined by day 25 following DOX treatment in the sham group **(red)** but was preserved with RIPC **(blue)**. **(C)** Cardiac global strain was not different between groups. No significant differences were reported in LV diameter in **(D)** diastole and **(E)** systole. **(F)** Cardiac output was higher in RIPC+DOX at 25 days. Data presented as mean ± SD. **Orange** = sham baseline; **green** = RIPC baseline; **red** = Sham + DOX; **blue** = RIPC + DOX. AA = anterior apical; AB = anterior basal; AM = anterior mid; PA = posterior anterior; PB = posterior basal; PM = posterior mid; other abbreviations as in Figure 1.

Mitochondrial respiration in both treatment groups was also similar to that in mitochondria from hearts of the naive cohort at 25 days following treatment.

RIPC ATTENUATES DOX-INDUCED APOPTOTIC AND AUTOPHAGIC SIGNALING. Cardiac expression of BCL2 and BAX, well-known indicators of apoptosis, was evaluated at 25 days following DOX and RIPC treatment. The results showed that BCL2 expression was markedly downregulated in DOX-treated mice ($p < 0.05$ vs. control subjects; $n = 3$) (Figure 6A), whereas RIPC preserved the expression of BCL2 ($p < 0.05$ vs. DOX). The expression of pro-apoptotic protein BAX was induced following DOX treatment, and it was not blunted by RIPC ($p > 0.05$ vs. control subjects; $n = 3$). Nevertheless, the significant 49% decline in BCL2/BAX ratio illustrated that DOX treatment enhanced pro-apoptotic signaling and that RIPC significantly restored the ratio to normal levels. Figure 6B demonstrates a significant decline in phosphorylated AKT1 by day 25 after DOX treatment, which was partially preserved with RIPC.

We further investigated and analyzed cardiac protein expression of Beclin (BECN1), one of the markers of autophagy, and our data indicated that it was induced by day 25 following DOX treatment ($p < 0.005$ vs. control subjects; $n = 3$) (Figure 7). Interestingly, RIPC prevented the DOX-induced increase in Beclin expression ($p < 0.005$ vs. DOX; $n = 3$). Moreover, the induction of the ratio of LC-3-II/I also confirmed the activation of autophagy with DOX treatment ($p < 0.05$ vs. control subjects), whereas RIPC effectively blunted the induction of the LCII/I ratio ($p < 0.05$ vs. DOX; $n = 3$). However, the expression level of p62, which is expected to be reciprocally regulated in relation to LC3, was not altered with DOX or RIPC.

DISCUSSION

Cardiotoxicity secondary to anthracyclines has been widely documented for the last 5 decades, but the mechanism remains poorly understood (10). Numerous studies have implicated oxidative stress by reactive oxygen species (ROS) (6), iron overload–induced toxicity (26), mitochondrial injury further perpetuating cardiac damage (7), and topoisomerase IIβ inhibition and DNA intercalation (4,5)

TABLE 1 Cardiac Physiological Parameters

	DOX (n = 18)		RIPC+DOX (n = 22)		p Value
	Baseline	25 Days	Baseline	25 Days	25 Days RIPC + DOX vs. 25 Days DOX
LV mass, mg	115.5 ± 3.9	97.8 ± 4.7	114.6 ± 5.7	105.1 ± 4.6	0.008
LVIDd, mm	4.50 ± 0.01	4.26 ± 0.06	4.38 ± 0.06	4.38 ± 0.09	NS
LVIDs, mm	3.17 ± 0.09	2.68 ± 0.11	3.08 ± 0.08	2.73 ± 0.11	NS
CO, ml/min	21.85 ± 1.12	22.63 ± 1.17	19.26 ± 0.97	25.97 ± 1.43	0.05
LV fibrosis, %	N/A	14.61 ± 1.83	N/A	10.60 ± 2.37	<0.001
Time points for LVEF, %					
Baseline	57.4 ± 1.2		55.4 ± 1.3		NS
1 week	61.4 ± 1.7		59.0 ± 3.8		NS
2 weeks	61.2 ± 1.6		62.3 ± 1.6		NS
3 weeks	60.6 ± 1.9		60.7 ± 1.8		NS
5 weeks	57.36 ± 4.9		57.4 ± 3.2		NS
9 weeks	59.7 ± 4.3		55.9 ± 1.7		NS
Segments at LAX longitudinal strain rate at 3 weeks ($Pk\%s^{-1}$)					
Posterior base	−9.3 ± 2.3		−8.5 ± 1.2		NS
Posterior mid	−7.2 ± 1.0		−5.1 ± 0.5		NS
Posterior apex	−7.4 ± 0.8		−5.9 ± 0.9		NS
Anterior apex	−9.0 ± 0.9		−8.6 ± 1.0		NS
Anterior mid	−8.3 ± 1.1		−5.6 ± 0.5		NS
Anterior base	−6.0 ± 0.9		−4.4 ± 0.7		NS

Values are mean ± SD.

CO = cardiac output; DOX = doxorubicin; LAX = long axis; LV = left ventricle; LVIDd = LV internal diameter diastole; LVIDs = LV internal diameter systole; RIPC = remote ischemic pre-conditioning.

among many other pathways as mechanisms that mediate DOX cardiotoxicity. However, therapeutic interventions individually targeting these pathways have not yielded much success in alleviating the clinical problem. Although limiting the cumulative dose of DOX, resorting to liposomal DOX, or use of dexrazoxane demonstrated some degree of success in attenuating cardiac injury, cardiovascular mortality due to cardiac toxicity in cancer survivors still contributes to morbidity and mortality in this patient population (27). Despite the significant improvements in developing new chemotherapeutic drugs as well as small molecule and biological treatment options that have expanded the life span of cancer survivors, anthracyclines are still a significant portion of the chemotherapy of choice for several cancers, including

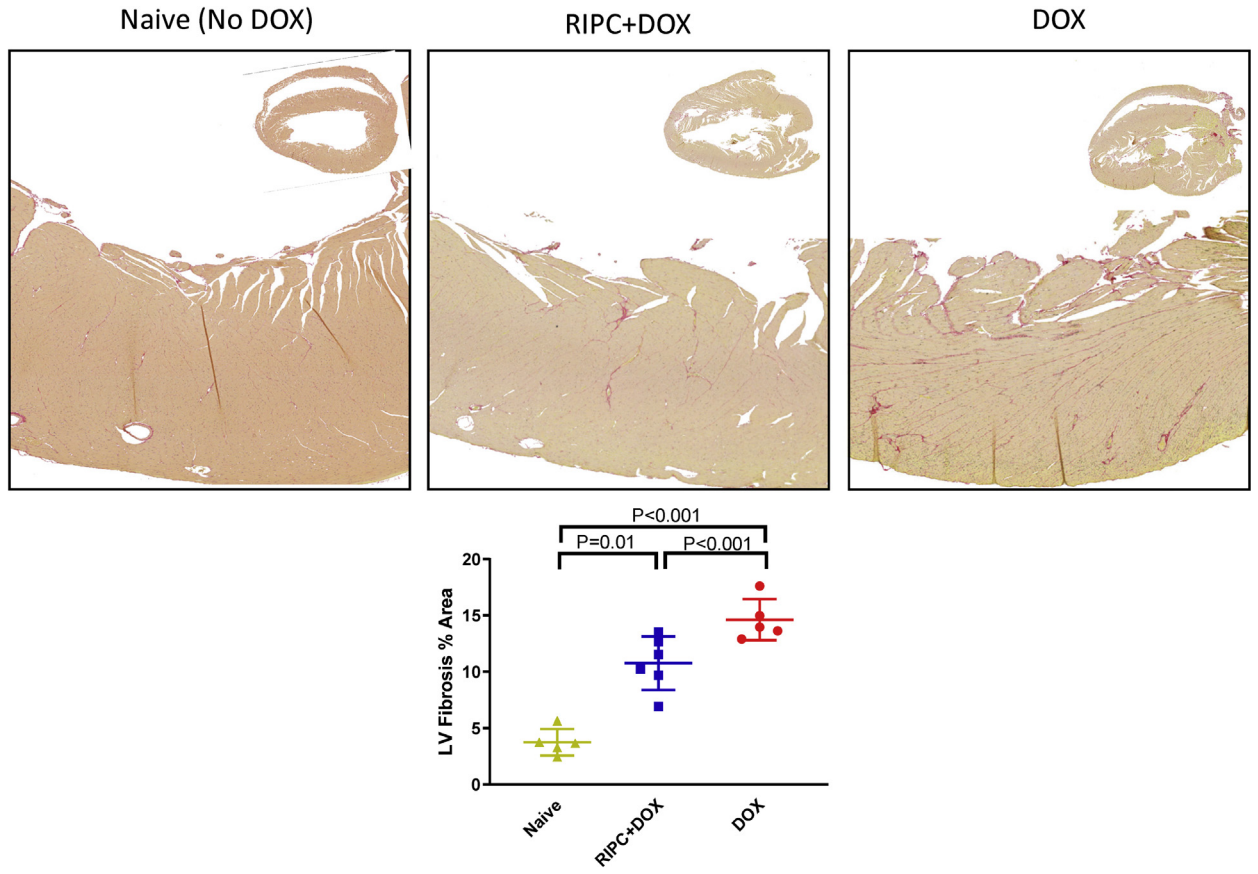
TABLE 2 Remote Pre-Conditioning Did Not Alter Respiration in Isolated Cardiac Mitochondria at 25 Days

	Control Naive (n = 5)	DOX (n = 6)	RIPC+DOX (n = 6)
Complex I substrates: glutamate + malate			
Protein yield (mg/g heart tissue)	27.4 (27.1–28.2)	27.2 (26.3–28.3)	26.2 (25.6–26.6)
State 3 (ADP stimulated)	443 (438.0–473.0)	507.5 (464.5–537.0)	446.0 (473.3–481.0)
State 4 (ADP limited)	54.0 (47.0–62.0)	62.0 (57.5–65.8)	61.5 (56.5–65.8)
RCR	8.0 (7.9–9.4)	8.0 (7.9–8.5)	7.7 (7.2–8.0)
2-mM ADP	604.0 (576.0–604.0)	596.5 (551.3–602.8)	522.0 (515.0–583.8)
DNP stimulated	567.0 (532.0–575.0)	574.0 (516.5–603.8)	503.0 (594.0–540.5)
Complex II substrates: succinate			
State 3 (ADP stimulated)	894.0 (854.0–923.0)	817.0 (795.3–862.0)	830.0 (787.0–875.3)
State 4 (ADP limited)	222.0 (213.0–240.0)	230.5 (186.5–255.0)	233.5 (218.3–244.3)
RCR	3.8 (3.8–3.9)	3.5 (3.3–3.6)	3.4 (3.3–3.6)
2-mM ADP	872.0 (806.0–872.0)	792.5 (729.8–815.5)	784.0 (711.5–846.8)
DNP stimulated	729.0 (715.0–758.0)	678.0 (602.8–720.3)	729.5 (650.5–803.3)

Values are median (interquartile range).

ADP = adenosine diphosphate; DNP = dinitrophenol; RCR = respiratory control ratio; other abbreviation as in Table 1.

FIGURE 4 Cardiac Fibrosis 25 Days Post-DOX Treatment

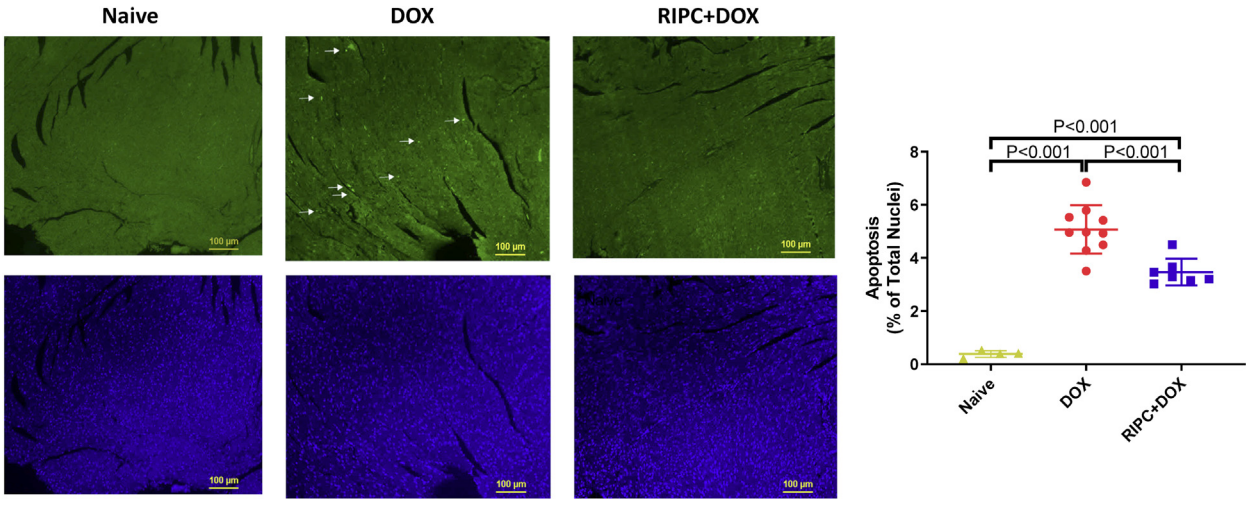


Picrosirius red staining of heart sections showed significant fibrosis by 25 days following DOX treatment compared with naive control hearts, which was significantly attenuated with RIPC. Data presented as mean ± SD. DOX **(red circles)**, RIPC+DOX **(blue squares)**, naive **(green triangles)**. Abbreviations as in **Figures 1** and **2**.

lymphoma in older adults, childhood cancer, and patients with breast cancer (28–31). This information motivates investigations into cardioprotective strategies that are multifaceted to encompass the known and rather unknown aspects and mechanisms of DOX cardiotoxicity (**Central Illustration**).

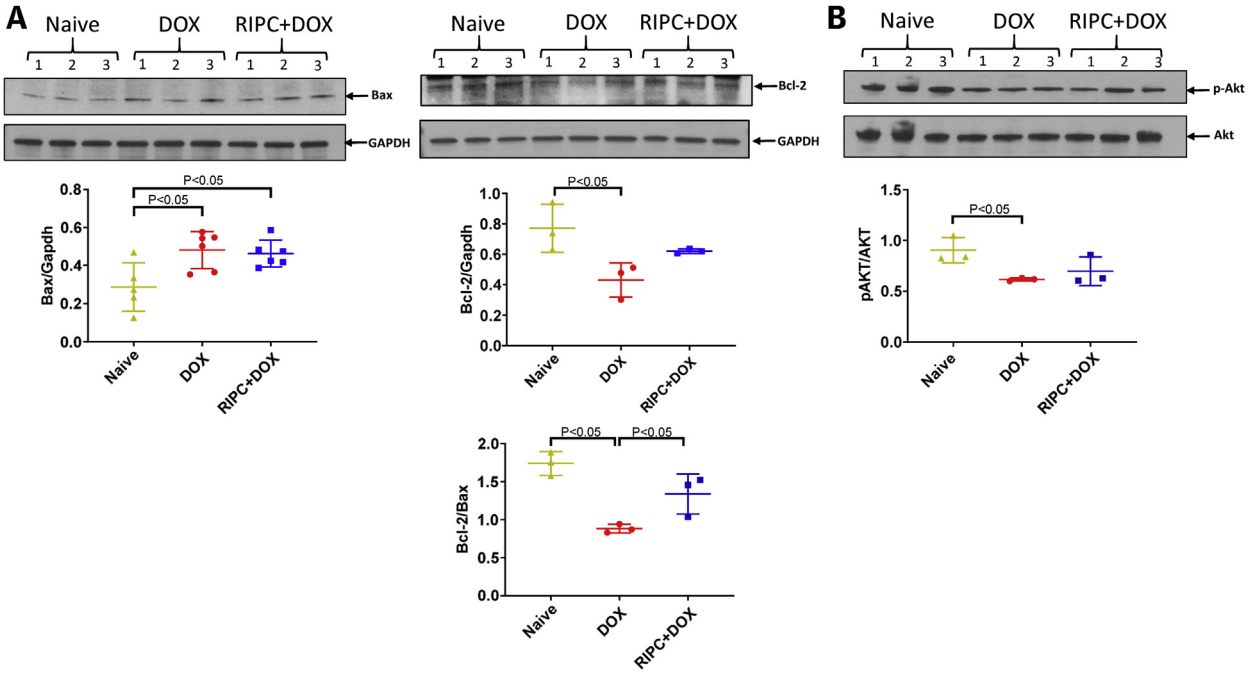
Myocardial ischemic pre-conditioning was first described by Murry et al. (11,12) in the mid-1980s and was inspired by the cardiac warmup phenomenon that cardiologists had been observing in patients with a history of angina who subsequently experienced myocardial infarction. The hypothesis behind the initial preclinical studies was that repeated cycles of short-lived or nonlethal ischemia and reperfusion by way of coronary artery occlusion and/or reflow would activate protective mechanisms that warn the heart about subsequent prolonged or lethal ischemia, thereby attenuating a host of pathological signaling that leads to oxidative stress, inflammation, mitochondrial dysfunction, and several modalities of cell death (32). This phenomenon was also shown to exert protective effects in other organs in the experimental setting, including the brain, liver, and kidneys. Not only was ischemic pre-conditioning protective when applied to the same organ that subsequently experienced sustained ischemia, but bouts of ischemia and reperfusion that affected a vascular bed remote from the heart, also known as RIPC, were shown to also protect the heart and other organs against sustained subsequent ischemia (14–17). The mechanism(s) of action of RIPC seem(s) to involve multiple pathways implicated in ischemic pre-conditioning in addition to some newly identified circulating factors, including exosomes and micro-RNAs (33–35). Mitochondrial damage during myocardial ischemia perpetuated the production of ROS at the onset of

FIGURE 5 Cardiac Apoptosis on Day 25 Following DOX Treatment



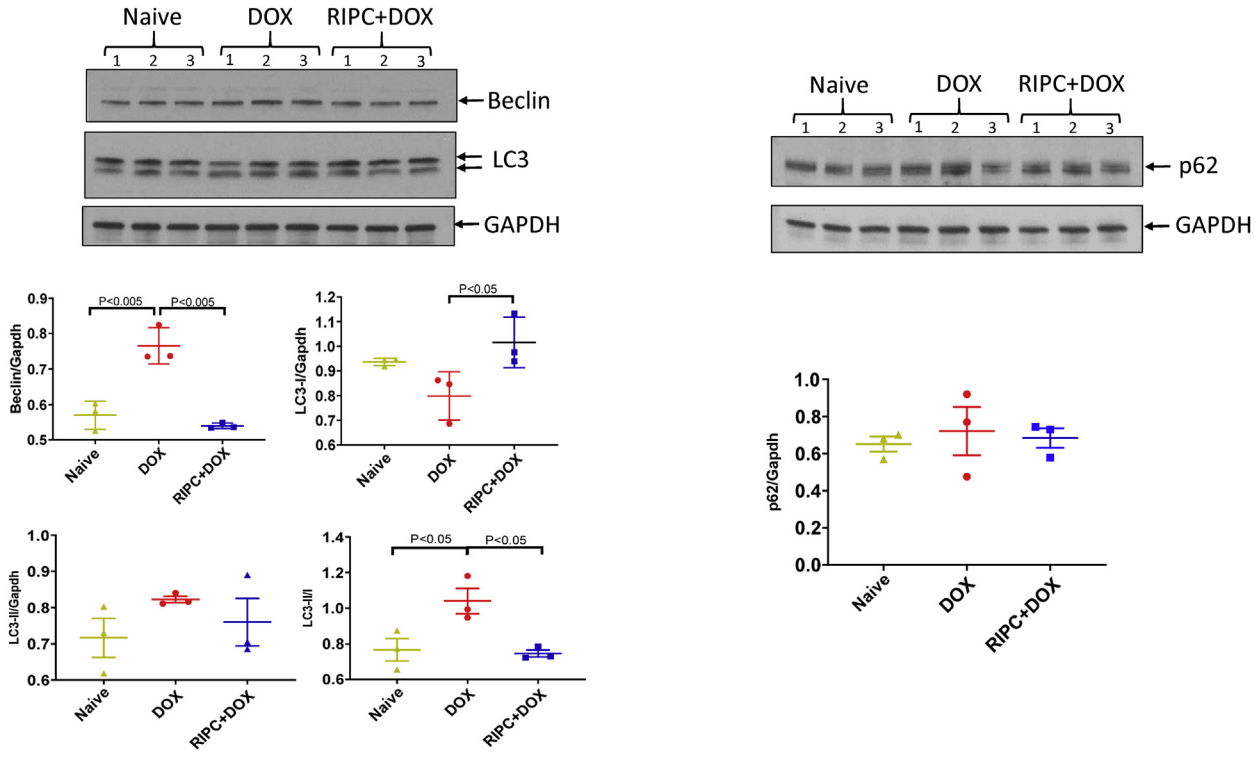
Terminal deoxynucleotidyl transferase dUTP nick end labeling staining demonstrating significant attenuation of cardiac apoptosis in RIPC+DOX group compared with DOX group, which was significantly higher than naive control hearts. Data presented as mean ± SD. DOX **(red circles)**, RIPC+DOX **(blue squares)**, naive **(green triangles)**. **Arrows** indicate apoptotic nuclei. Groups as noted in Figure 1.

FIGURE 6 Cardiac Proapoptotic and Antiapoptotic Protein Expression and AKT1 Survival Signaling 25 Days Following DOX Treatment



(A) Western blot analysis revealed a significant decline in BCL2/BAX ratio with DOX, indicating pro-apoptotic signaling that was blunted with RIPC. **(B)** Pro-survival AKT1 phosphorylation was also significantly decreased with DOX but was partially preserved with RIPC. Data presented as mean ± SD. DOX **(red circles)**, RIPC+DOX **(blue squares)**, naive **(green triangles)**. Abbreviations as in Figure 1.

FIGURE 7 **Autophagy Signaling in the Heart 25 Days Post-DOX Administration**

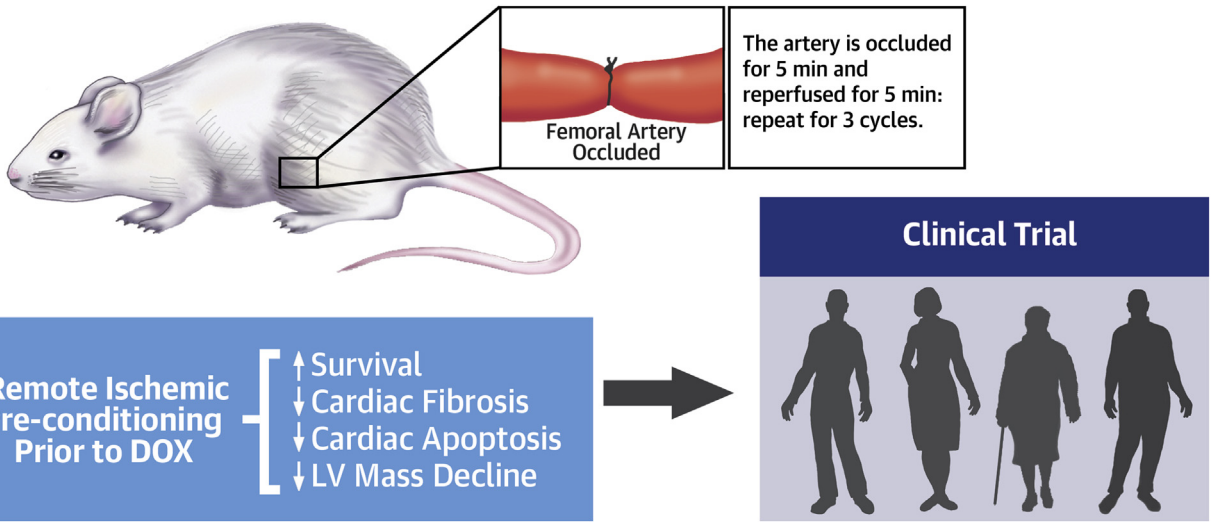


Autophagy markers Beclin (BECN1) and LC3-II/I were significantly increased by day 25 after DOX treatment, which was abrogated with RIPC. No changes were observed in p62 expression at this time point. Data presented as mean ± SD. DOX **(red circles)**, RIPC +DOX **(blue squares)**, naive **(green triangles)**. Abbreviations as in Figure 1.

reperfusion due to damage incurred in the electron transport chain (36). Ischemic pre-conditioning, due to upregulation of several antioxidant proteins, was effective in attenuating oxidative stress and reducing cardiac injury following ischemia (37). Among several observed modalities of cell death in the context of ischemia–reperfusion injury and DOX cardiotoxicity, ferroptosis was a key mechanism downstream of excessive ROS production and iron overload (26). ROS-induced oxidation of membrane lipids was shown to increase free iron levels—an effect that was mitigated by pre-conditioning before inducing ischemia/reperfusion in rats (38).

Because of the similarity of pathological signaling between ischemia/reperfusion injury and DOX cardiotoxicity, the present study evaluated the potential protective effect of RIPC against DOX cardiotoxicity in mice (37,39). Our data demonstrated significant reduction in mortality up to 85 days post-DOX administration ($p = 0.007$), which was associated with attenuation of a decrease in LV mass ($p = 0.008$), despite a lack of significant changes in LVEF. DOX-induced cardiac fibrosis and apoptosis were also significantly mitigated by RIPC ($p < 0.05$ vs. DOX). These changes were also accompanied by attenuation of apoptosis, as well as autophagy signaling markers with RIPC compared with DOX. The persistent increase in autophagy markers by day 25 following DOX treatment could indicate enduring damage that necessitates removal of impaired components by the cell, which was not needed in the RIPC cohort. The attenuation of cardiac fibrosis and apoptosis indicated prevention of different modalities of cell death, which was suspected to occur over time and ultimately led to cardiomyopathy. Because apoptosis is the predominant method of cell death with DOX, our data, which demonstrated a decline in LV mass with DOX, were in line with clinical data in the literature and the ability of RIPC to mitigate apoptosis. Its signaling might explain the preservation of LV mass with this treatment (40). Although DOX-induced mitochondrial dysfunction was implicated in several previous studies, our results suggested that RIPC did not markedly affect mitochondrial function in the DOX-treated mice, at least at the time point considered in this study, which only took into account mice that survived for 3 weeks after DOX treatment. More comprehensive assessment of mitochondrial function

CENTRAL ILLUSTRATION **Remote Ischemic Pre-Conditioning Against DOX Cardiotoxicity**



Gertz, Z.M. et al. J Am Coll Cardiol CardioOnc. 2019;1(2):221-34.

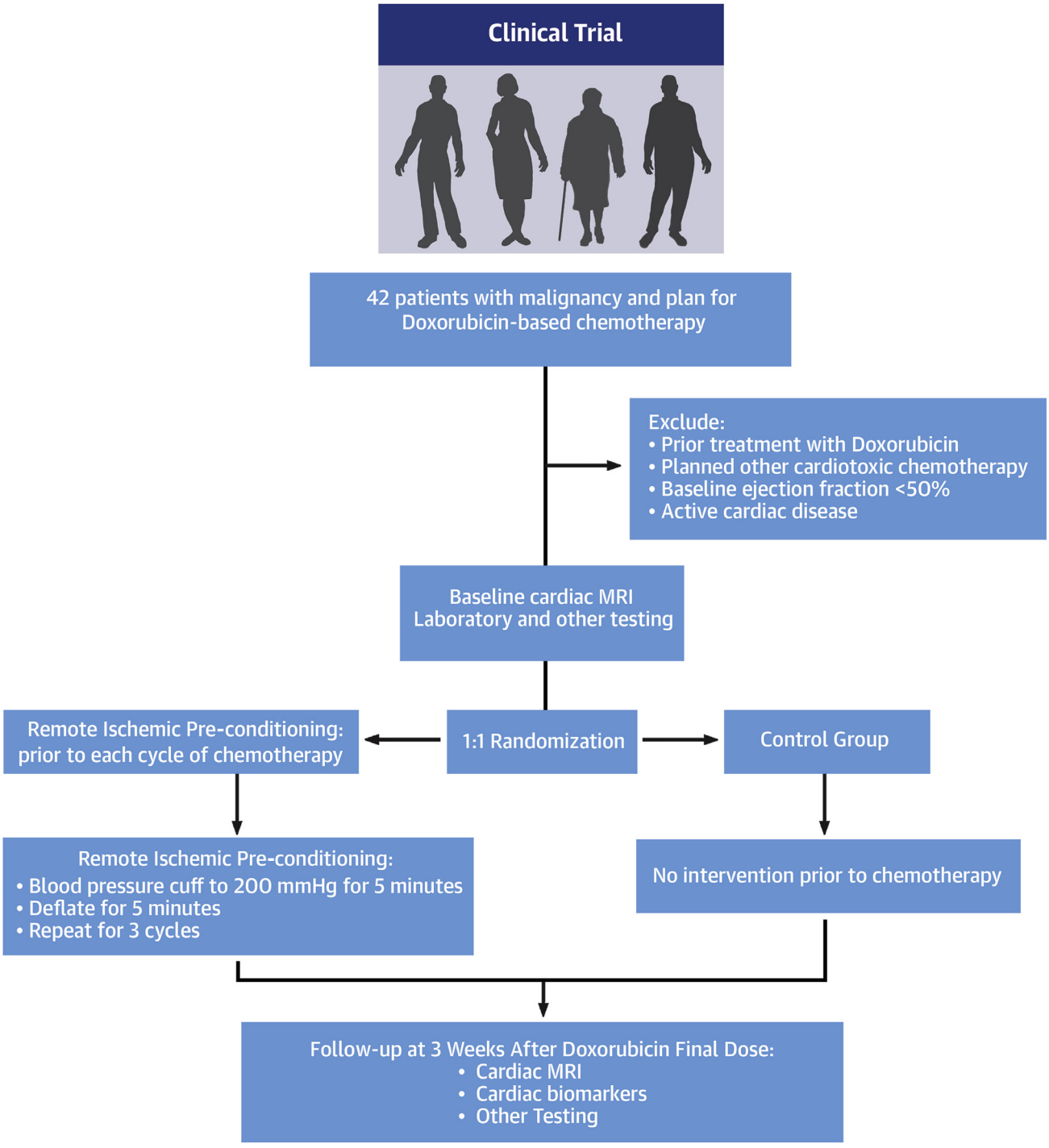
Key outcomes of improved survival, attenuation of left ventricular (LV) mass decline and mitigation of fibrosis. DOX = doxorubicin.

at earlier time points following DOX treatment is warranted.

Several studies in the literature evaluated the potential benefits of RIPC in clinical settings, which were recently summarized in a meta-analysis of randomized trials on remote ischemic conditioning (RIC) during primary percutaneous coronary intervention in patients with ST-segment elevation myocardial infarction (STEMI) (41). The analysis included 8 randomized trials with 1,083 patients with STEMI who underwent primary percutaneous coronary intervention with RIC and standard of care versus percutaneous coronary intervention with standard of care only. RIC significantly reduced infarct size measured by biomarker release ($p = 0.001$), improved ST-segment resolution ($p < 0.001$), attenuated major adverse cardiac and cerebrovascular events ($p = 0.003$), and caused a nonsignificant decrease in infarct size assessed by cardiac imaging ($p = 0.36$). Because most of the evaluated outcomes favored RIC, the investigators concluded that RIC might improve cardiovascular outcomes in patients with STEMI. In addition to the potential benefits of RIPC in the context of myocardial ischemia, sepsis (35), and coronary artery bypass graft surgery (42), preclinical studies also indicated that ischemic pre-conditioning protected cardiomyocytes against DOX-induced toxicity through a mechanism involving PI3K (39). Our data indicated a decrease in cardiac AKT1 phosphorylation in the DOX-sham group, which was partially preserved with RIPC. In addition, several mechanisms that were shown to mediate DOX toxicity in the heart and cardiomyocytes were also regulated by RIPC, including micro-RNA-21, soluble guanylate cyclase activity, protein kinase G, and Sirt3, among others (43-46).

This knowledge led to the design of the ERIC-ONC (Effect of Remote Ischemic Conditioning in Oncology; NCT02471885) trial (47), which is ongoing. This is a single-center, blinded, randomized, sham-controlled study that aims to enroll 128 adult oncology patients who will receive anthracycline-based chemotherapy. The patients will be randomized in a 1:1 ratio to sham or RIPC with 4 cycles of 5-min upper arm blood pressure cuff inflation and/or deflation immediately before receiving chemotherapy throughout the treatment regimen. The primary endpoint will be high-sensitivity troponin T levels over 6 cycles of chemotherapy with follow-up at 12 months. Secondary endpoints will include clinical, electrical (incidence of arrhythmias), structural (echocardiography to assess function), and biochemical parameters (mitochondrial DNA, micro-RNA, and proteomics). This effort is among the few nonpharmacological attempts to combat DOX cardiotoxicity. Other clinical trials with pharmacotherapy-based approaches to date included the randomized controlled CECCY (Carvedilol Effect

FIGURE 8 **RIPC Against DOX Cardiotoxicity: Clinical Trial Design**



The design of the clinical trial is also included to demonstrate the translational bench-to-bedside efforts at Virginia Commonwealth University. MRI = magnetic resonance imaging.

in Preventing Chemotherapy-Induced Cardiotoxicity) trial, which involved 200 patients and demonstrated a significant reduction in diastolic dysfunction and troponin I levels over time in breast cancer patients who were treated with carvedilol while receiving anthracycline chemotherapy (48). The OVERCOME (Prevention of the Left Ventricular Dysfunction With Enalapril and Carvedilol in Patients Submitted to Intensive Chemotherapy for the Treatment of Malignant Hemopathies) trial tested the combination of angiotensin-converting enzyme inhibitors and carvedilol in 90 patients. This trial took advantage of functional assessment using cardiac magnetic resonance in addition to transthoracic echocardiography as a primary endpoint (49). The outcomes of this study indicated that prompt combined treatment with angiotensin-converting enzyme inhibitors and ß-blockers might prevent LV systolic dysfunction in patients with malignant hemopathies who receive intensive chemotherapy. In this context, an interesting study by Cardinale et al. (50) demonstrated that prompt initiation of treatment played an important role in the extent of LV functional recovery. A remarkable observation in this study was that most (98%) cases associated with significant decline in LV function (reduction in LVEF between 10% and 50%) occurred in the first 12 months after DOX treatment, which highlighted the importance of early detection and timely intervention for best functional recovery outcomes. This group also noted that strategies that targeted prevention of DOX cardiotoxicity would be preferred, rather than treatment of an established cardiomyopathy that is progressive and may less likely be reversible after surpassing a certain threshold (51). To this end, the application of RIPC immediately before DOX administration, as was the case in the present study, might prove more promising than interventions that are applied later in the course of cardiac dysfunction. Moreover, RIPC is a noninvasive and safe approach that has been shown to protect multiple organs, including the heart against severe ischemia and lung injury after pulmonary resection in cancer patients (15,52).

STUDY LIMITATIONS. Our study provided proof of concept that supported the notion of cardioprotection against DOX toxicity with RIPC. Despite the benefits reported, there remain some limitations with regard to the intraperitoneal administration of a single high dose of DOX in our study. Although this administration route did not resemble the clinical approach, it was consistent with numerous preclinical studies in mice and rats, and therefore, was helpful for comparison with other study outcomes. We decided to apply RIPC 1 h before DOX injection based on the known window of protection offered by RIPC and the practicality or feasibility of this time point in the clinical setting. However, further studies are needed to refine the time of intervention, because the efficacy of RIPC was documented starting from 5 min and lasting up to 72 h (53,54).

Another limitation was that our study focused on male mice that were healthy (i.e., free of cancer and cardiovascular risk factors). This was particularly important because RIPC failed to reduce myocardial infarct size in the Zucker fatty rat model of type 2 diabetes due to a lack of humoral communication (55). This might have important implications for cancer patients with type 2 diabetes and warrants more in-depth research that also includes female mice, cancer models, and cardiovascular risk factors. Studying the effects of RIPC in a tumor-bearing mouse model is also needed to provide in-depth understanding regarding the effects of RIPC not only on the heart, but also on DOX efficacy, if any.

CONCLUSIONS

The application of RIPC 1 h before DOX administration in adult male mice attenuated mortality, LV mass decline, cardiac fibrosis, apoptosis, and autophagy signaling. These findings suggest that, with further study, RIPC might prevent or attenuate DOX cardiotoxicity and provide an affordable and noninvasive cardioprotective strategy. The impact of DOX on cardiac mitochondria should be further addressed with evaluation of mitochondrial oxidative phosphorylation and overall integrity at earlier time points in DOX-treated animals. Based on our data and the findings reported in the literature, we designed a single-center clinical trial to test RIPC for prevention of anthracycline-related cardiotoxicity (**Central Illustration**, **Figure 8**). In terms of patient selection, chemotherapeutic regimen, and RIPC protocol, our study design was similar to the ERIC-ONC study (47). However, although the ERIC-ONC study proposed to use cardiac troponin change as the primary outcome, our study uses cardiac magnetic resonance imaging to evaluate changes in LV function as the primary outcome (56). Its high reproducibility makes it an excellent modality for monitoring response to protective interventions against chemotherapy-induced cardiotoxicity (57), such as that in our proposed trial.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: The use of DOX has been limited by dose-dependent cardiotoxicity and heart failure. Because of the multifactorial beneficial signaling pathways elicited by RIPC, we tested the application of RIPC before DOX administration to prevent cardiotoxicity. In adult male mice, survival was significantly improved, and LV mass decline, cardiac fibrosis, apoptosis, and autophagy signaling were attenuated.

TRANSLATIONAL OUTLOOK: The potential benefit of RIPC before DOX administration in preventing DOX cardiotoxicity is being tested in human clinical translational studies, including the ongoing ERIC-ONC trial and our upcoming clinical trial.

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KEY WORDS apoptosis, cardiac fibrosis, doxorubicin, echocardiography, remote ischemic pre-conditioning

APPENDIX For an expanded Methods section, please see the online version of this paper.