Evaluation of Doxorubicin vs. Aclarubicin Exposure in C57BL/6J Mice as a Chronic Model of Anthracycline Induced Cardiotoxicity

Darleen Song Tu

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Committee:

Brian M. Iritani

Elizabeth R. Lawlor

Kimberly S. Waggie

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Abstract

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Darleen Song Tu

Chair of the Supervisory Committee:
Brian M. Iritani
Department of Comparative Medicine

Doxorubicin is an anthracycline chemotherapeutic limited by dose-dependent cardiotoxic effects, while Aclarubicin is a promising alternative anthracycline shown to be less cardiotoxic. Future clinical trials involving Aclarubicin will need to be tested in a relapse setting where patients have already received Doxorubicin. The study's objective was to evaluate the cardiac effects of Aclarubicin following administration of Doxorubicin and develop a standardized mouse model for assessing cardiotoxicity of oncologic adjuvants after prior Doxorubicin exposure. C57BL/6J mice all received an initial once weekly dose of 5 mg/kg intraperitoneal injections for 4 weeks and rested for an additional 4 weeks. Mice were then administered intraperitoneal injections of one of the following agents once-a-week for 4 weeks: Doxorubicin

(5 mg/kg), Aclarubicin (5 mg/kg) or equivalent volumes of saline. Mice were humanely sacrificed after an additional 12-week rest period. Mice that received 40 mg/kg cumulative Doxorubicin had the lowest survival rate and the most weight loss. There were no detectable echocardiographic measurement differences between groups. Overall cardiac changes for all three mice groups were mild via histologic examination by periostin immunohistochemistry, trichrome, and hematoxylin and eosin staining. The results indicate Aclarubicin does not further potentiate Doxorubicin cardiotoxicity.

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ACKNOWLEDGEMENTS

The author thanks our collaborators Jacques Neefjes PhD, Head Department Cell and Chemical Biology, at Leiden University Medical Center and their laboratory team for compounding Aclarubicin for the study. This study was supported by grant funding provided by CURE Childhood Cancer and NIH NCI. The Microscopy and Histopathology CoLab at Seattle Children's Research Institute performed whole slide scans. Figures created with BioRender.com.

DEDICATION

This work is dedicated to my partner, my family and all my mentors that have guided me along this academic journey.

LIST OF ABBREVIATIONS

Doxo, Doxorubicin

Acla, Aclarubicin

IP, intraperitoneal

D-Saline, Doxorubicin-Saline treatment group

D-Doxo, Doxorubicin-Doxorubicin treatment group

D-Acla, Doxorubicin-Aclarubicin treatment group

H&E, Hematoxylin and eosin

Echo, Echocardiogram

MV E, Early diastolic trans-mitral flow velocity

MV A, Late diastolic trans-mitral flow velocity

INTRODUCTION

Doxorubicin (Doxo) is an anthracycline chemotherapeutic used to combat a majority of cancers, however, as the lifetime expectancy of these cancer survivors treated with Doxo have lengthened, long term adverse dose-dependent cardiotoxic effects associated with Doxo administration have become well documented.[5] Aclarubicin (Acla) is a promising alternative chemotherapeutic that demonstrates anti-tumor efficacy without associated cardiotoxicity, despite being classified as an anthracycline like Doxo.[12, 18]

Several mechanisms have been suggested to cause Doxo cardiotoxicity; activation of apoptotic pathways, production of radical oxygen species, and disruption of iron metabolism are a few examples.[19] Recent literature posited Doxo cardiotoxicity might be linked to the drug's combined effects on histone eviction and Topoisomerase II poisoning leading to double stranded DNA breaks.[24] Furthermore, Acla has been proposed to maintain anti-tumor efficacy while reducing cardiotoxicity through mechanisms on histone eviction without Topoisomerase II poisoning.

Doxo cardiotoxicity has an acute or chronic onset; chronic cases can occur within a year of treatment or even many years afterwards.[25] Chronic Doxo cardiomyopathy can cause dilation in the four chambers of the heart and result in the decline of ventricular ejection fraction.[7] There are few effective treatments available once patients develop anthracycline induced cardiotoxicity, stressing the imperative to seek less cardiotoxic alternatives.

Acla is not a U.S Food and Drug Administration approved drug and future clinical trials involving Acla will need to be tested in settings where relapsed patients have typically received threshold dosages of Doxo. There is no existing literature that investigates whether administration of Acla following Doxo will potentiate cardiotoxicity, nor is there a published C57BL/6 mouse model assessing chemotherapeutics after an initial exposure to Doxo. This paper provides reference values for future studies using hematologic, echocardiographic (echo), and histologic analyses.

Many different mouse models studying chronic Doxo cardiotoxicity exist; all utilizing different doses, frequency, and duration of the medication.[17] Recent studies with reported cardiotoxicity have used a 4-5 mg/kg/dose, repeated for a cumulative dose of 20-25 mg/kg, and evaluated hearts between 1-13 weeks out from final dose.[3, 9, 28] Having a commonly used chronic Doxo mouse model with reproducible and clinically relevant severity of cardiotoxicity is valuable for preclinical research.

Our objective is to develop a protocol that can serve as a standardized mouse model for assessing the cumulative cardiotoxic or cardioprotective effects of new oncologic adjuvants that will be prescribed for patients with cancer who relapse after prior Doxo exposure. Here we specifically evaluate and compare the cardiac effects of Acla and Doxo in mice that have previously received a moderately cardiotoxic Doxo regimen. We hypothesized that exposure to Acla following an initial Doxo administration will not induce further cardiotoxicity compared to a regimen of additional Doxo in C57BL/6J mice.

MATERIALS AND METHODS

Animals. All experimental procedures adhere to the *Guide for the Care and Use of Laboratory Animals* and were approved by Seattle Children's Research Institute IACUC.[13] The institute is fully AAALAC-accredited and has a Public Health Service approved Animal Welfare Assurance.

A total of 40 male C57BL/6J (7-weeks-old) mice were purchased from Jackson Laboratory (JAX stock #000664, Sacramento, CA) and acclimated to study for 1 week. All mice were housed in the Seattle Children's Research Institute vivarium (68-79°F, 30-70% humidity, 12:12 light cycle 7:00-19:00). Mice were socially housed in a group of 5 mice per Allentown ventilated micro-isolation caging (Allentown, NJ) and provided standard PMI PicoLab Rodent 5053 irradiated chow ad libitum and chlorinated automatic water. Cage, corn cob bedding, nestlet and crinkle paper enrichment were autoclaved before use. Cage changes were performed every two weeks and spot cleaned as needed. Mice were specific pathogen free for the following excluded microbial agents: Ectromelia virus, Mouse rotavirus, Lymphocytic choriomeningitis virus, M*ycoplasma pulmonis*, Mouse hepatitis virus, Mouse parvovirus, Minute virus of mice, Pneumonia virus of mice, Reovirus-3, Theiler's murine encephalomyelitis virus, Sendai virus, pin worms and fur mites.

Treatment. Doxo (353, Millipore Sigma) and Acla (Neefjes, Leiden, Netherlands) preparations were reconstituted with 0.9% sterile saline to a 0.5mg/mL solution.

The study design is depicted in Figure 1. Mice (n = 40) were enrolled into the study at 8 weeks of age and began Doxo exposure at 5 mg/kg/dose intraperitoneal injections (IP) once weekly for 4 weeks. Mice underwent a rest period of 4 weeks after receiving a cumulative dosage of 20 mg/kg of Doxo and randomized into three experimental groups D-Saline (n = 10), D-Doxo (n = 15), or D-Acla (n = 15) at the end of the rest period. During the second exposure mice were administered treatment for an additional 4 weeks by their respective group: Doxo (5 mg/kg), Acla (5 mg/kg), or equivalent volume of saline IP, once weekly.

Mice had the following cumulative dosages: D-Saline mice received 20 mg/kg of Doxo, D-Doxo mice received 40mg/kg of Doxo, and D-Acla mice received 20 mg/kg of Doxo followed by 20mg/kg of Acla.

Mice were monitored three times a week on non-consecutive days for early end-point criteria which included signs of morbidity (ex. lethargy, dyspnea) or weight loss beyond 15% body weight. All mice were humanely sacrificed by carbon dioxide asphyxiation at the end of study.

Echocardiogram. VEVO 3100 High-Resolution Micro-Ultrasound System (VisualSonics Inc., Toronto, Canada) was used for echo measurements under isoflurane anesthesia at a maintenance of 1-2%. Heart rates were maintained between 400-600 beats per minute and heat support was provided to maintain body temperature at 37°C. Echos were performed by a cardiologist blinded to treatment groups.

Measurements were performed on five mice from each group before the start of the second exposure and 12 weeks after the final exposure. When possible, an early end-point echo was obtained for any mice with mortality before scheduled end point.

Hematologic analysis. Prior to euthanasia, all mice had two drops of blood collected awake by submental lancet puncture or via retroorbital bleeding under isoflurane anesthesia (2% maintenance). Complete blood counts were collected via two drops of blood into EDTA coated tubes and were analyzed using ElementHt5 (Heska, Loveland, CO).

For serum analysis, blood was collected by retroorbital bleeding under isoflurane anesthesia (2% maintenance) at the end of study. Blood was allowed to coagulate for 20-45 minutes and spun at 2500 RPM for 15 minutes. Isolated serum was frozen at -20°C and submitted to IDEXX (Sacramento, CA).

Tissue Preparation. Heart, lung, kidney, and liver were weighed, and tibial length measured during tissue collection. The formula (heart weight ÷ tibial length^3) was utilized to index heart weights to normalize for mouse variation in body size.[8] Remaining organs were indexed with the formula (organ weight ÷ tibial length). Hearts were fixed in 10% neutral buffered formalin and paraffin embedded sections cut to 2 µm thickness in a four-chamber view. Sections were stained with hematoxylin and eosin (H&E) to be evaluated for vacuolization and trichrome special stain to highlight fibrosis. A peroxidase-based detection system (B40925, Thermofischer) and ImmPact DAB Substrate Kit (SK-4105, Vector Laboratories) was used to perform immunohistochemistry with periostin primary antibody (diluted 1:100, ab215199,

Abcam) and Rabbit IgG (diluted 1:100, 02-6102, Invitrogen) as a negative control. Whole slides were scanned with ZIESS Axioscan 7 (White Plains, NY) and histologic images captured using Qupath (version 0.5.1).[4]

Histology Analysis. A veterinary pathologist blinded to treatment groups evaluated whole heart sections and scored ventricles by severity of vacuolization, fibrosis, and immunohistochemistry stain-uptake (Table 1&2). Three areas (right ventricle, left ventricle, and interventricular septum) received a score and were summed for each heart. Scores were averaged by treatment group and calculated for statistically significant differences.

Statistical analysis. Graphpad Prism (version 10.2.3) was used for statistical analysis. Kaplan-Meier Curves were analyzed using Log-rank (Mantel-Cox) test. Student's T-test was used to determine significant differences between two groups and One-way ANOVA with Tukey's Multiple Comparison for any group analysis greater than two. Statistical significance was determined by a P value of ≤ 0.05.

RESULTS

Survival and weights. Eleven D-Acla mice from the initial 15, 5 of 10 D-saline mice, and 3 of 15 D-Doxo mice survived to the end of study (Figure 2). D-Doxo mice had significantly lower survival compared to D-Acla mice. No other statistically significant differences were detected. Mice from all three groups were euthanized for early mortality starting at week 10. Median survival was greater in D-saline mice at 20.5 weeks compared to D-Doxo at 12 weeks (Table 3). D-Acla mice survived at rates greater than 50% through the study and so median survival remained undefined.

All mice euthanized for early mortality reached end point criteria of 15% weight loss (Table 4). Between weeks 15-17, three D-saline mice presented hunched or minimal to mildly lethargic; one mouse was mild to moderately pale. One D-Doxo mouse presented moribund with mild pallor at week 21.

At the end of the first Doxo exposure all mice had lost weight with no significant differences between groups (Figure 3A). D-Doxo mice had significantly greater weight loss by the completion of the second exposure period (week 11) compared to both D-Acla and D-Saline mice. D-Acla mice were first to regain average weight above baseline at week 12, while D-saline mice gained above baseline by week 14, and D-Doxo by week 18. Heart weights were not significantly different between treatment groups regardless of early mortality or survival to end of study (Figure 3B). Lung, kidney, and liver weights were not significantly different between

treatment groups at the end of study (Figure 3C). Kidney and liver weights are provided in supplemental materials.

Echocardiographic measurements. Echo measurements of mice at baseline (after completion of initial Doxo exposure and before second exposure period), D-saline, D-Doxo, and D-Acla were compared (Figure 4A-E). Heart rate was significantly lower in D-Doxo early mortality mice compared to baseline, D-Doxo survivor, and D-Acla survivor measurements. Ejection fraction and fractional shortening was not significantly different between treatment groups. D-Doxo early mortality mice had lower early diastolic trans-mitral flow velocity (MV E) compared to baseline, D-Saline survivors, and D-Acla survivors. D-Acla survivor mice had significantly higher late diastolic trans-mitral flow velocity (MV A) than D-Doxo and D-Saline mice. Remaining echo measurements are reported in the supplemental materials.

Hematologic analysis. The full complete blood count and serum chemistry panel can be referenced in the supplementary materials.

Complete blood count. Total leukocyte and lymphocyte counts were significantly lower in D-Doxo early mortality mice compared to D-Acla mice that survived to the end of study (Figure 5A-F). Lymphocyte counts were higher in all survivor groups compared to D-Saline early mortality and D-Doxo early mortality mice. Neutrophil, eosinophil, erythrocyte, and platelet counts had no significant differences between all treatment groups regardless of early mortality or survival to end point.

Serum chemistries. Calcium was significantly lower in D-Doxo survivor mice compared to D-Acla survivor mice (Figure 6A-F). Phosphorous was significantly lower in D-Saline survivor mice compared to mice that received additional anthracyclines. No significant differences were seen between potassium, cholesterol, glucose, and creatinine kinase serum concentrations.

Histology. No significant differences were detected by scoring for H&E, trichrome, and periostin (Table 5). All hearts contained minimal to mild vacuolization, fibrosis, and positive staining for periostin (Figure 7-9). Observed lesions were focal or multifocal in distribution.

DISCUSSION

As hypothesized, administration of Acla after exposure to Doxo in mice did not potentiate cardiotoxicity. D-Acla mice had significantly higher survival rates compared to D-Doxo mice. D-Doxo mice had significantly lower weights by the end of the second exposure period compared to the other treatment groups. Overall cardiac changes for all three mouse groups were similar via histologic examination. All hearts had minimal to mild, focal to multifocal vacuolation of cardiomyocytes, fibrosis, and positive periostin staining. However, echo ejection fraction and fractional shortening measurements and lesions on histology were not as profound as published in previous literature.[3, 29]

D-Doxo mice had significantly lower survival rates compared to D-Acla mice. Though not statistically significant, but clinically relevant, D-Acla mice had 23% greater survival than D-saline mice and D-saline mice had 30% greater survival than D-Doxo mice. Each treatment group had early mortality by week 10, which was 6 weeks after completing the first Doxo exposure and after mice had received an additional two doses of treatment for the second exposure period. The early mortality of even D-Saline mice indicates the initial exposure of a 20 mg/kg cumulative Doxo dose was toxic; additional saline injections would not have caused mortality in D-Saline mice.

However, adverse effects from additional anthracycline administration have not been ruled out as there was a higher degree of mortality in the D-Doxo and D-Acla group at week 10. Groups that received additional Acla had higher survivability with no other mortalities beyond

week 10. Additional Doxo administration after completion of the first Doxo exposure was not well tolerated in D-Doxo mice; mortality was highest half-way through the second exposure period when mice had received a cumulative dose of 30 mg/kg of Doxo and continued until a week after treatment. D-Doxo mice had significantly lower weights by the end of the second exposure period and had prolonged weight loss compared to the other treatment groups.

Some functional parameters of the heart were significantly lower in D-Doxo early mortality mice as well. All survivor groups had higher heart rates compared to D-Doxo early mortality mice. D-Doxo early mortality mice might not have been able to support higher heart rates while under isoflurane anesthesia during echos. D-Doxo early mortality mice also had reduced diastolic functional measurements (MV E) compared to other treatment group survivors, but no differences in ejection fraction or fractional shortening.

Most literature refers to ejection fraction and fractional shortening as clinical indicators of chemotherapeutic associated cardiotoxcity.[10] When comparing our experimental mice echo measurements to normal reference values seen in experimentally naïve C57BL/6 mice (represented by the shaded regions of Figure 4A-E) 26% of baseline echos were lower than the normal reference values for ejection fraction and fractional shortening.[27] Treatment groups were on average within reference range; with some mice measuring mild to moderately below. Echo measurements performed in the remaining three D-Doxo survivor mice might have selected for D-Doxo mice resistant to cardiotoxicity; their ejection fraction and fractional shortening was within normal reference ranges. D-Doxo mice with baseline measurements had not survived through to week 24 of study and a comparison of changes from early mortality measurements

could not be made against D-Acla survivor mice. The low power when comparing early mortality and survivor mice might complicate the statistical findings we see in this study.

Cardiotoxicity in human patients is defined by the American Society of Echocardiography as left ventricular ejection fraction measurements of <50-53% or a 10% reduction from baseline.[6] There is not a similarly defined parameter for cardiotoxicity in mice. In the literature, chronically treated Doxo mice receiving a cumulative dose of 20 mg/kg (4 mg/kg weekly for 5 weeks) demonstrated ejection fractions which remained unchanged one week after completing Doxo exposure and saw an ejection fraction reduction of greater than 30% on average after 7 and 11 weeks.[14] Mice given a cumulative dose of 25 mg/kg of Doxo (5 mg/kg every seven days for 5 weeks) had ejection fraction reductions of 32% from baseline at the end of week 6.[21] The above models reported cardiotoxicity by echo at lower cumulative doses and in shorter time frames; D-Doxo mice in our study were administered nearly twice as much for the cumulative dose. Our mice were on study 20 weeks out from the initial Doxo exposure and 12 weeks out from the second anthracycline exposure. There were no significant differences in ejection fraction and fractional shortening, however baseline echos in this study were taken after mice had received the first Doxo exposure. Greater changes could have been detected if initials echos were performed prior to any exposure.

Hematologic analysis was conducted since echo changes were not as severe as expected. Experimental mice had alterations of white blood cell, red blood cell, and platelet counts compared to published reference ranges (represented by the shaded regions of Figure 5A-F) [1, 22]. D-Doxo early mortality mice presented with a leukopenia, lymphopenia, neutrophilia, and

eosinopenia. D-Saline early mortality and D-Acla early mortality mice followed a similar trend. The leukogram reflects either a stress or inflammatory pattern. Mice from all treatment groups regardless of survivor status also had elevated platelets on average; a thrombocytosis can be suggestive of increased inflammation as well. Erythrocyte counts were within reference ranges for all groups on average. A subset of mice from each group were reported with mild to severe anemia. Chemotherapeutic bone marrow depletion could explain the leukopenia and anemia seen, but mice sampled further out from exposures of anthracycline are less likely to be affected by this cause.

Serum chemistries in survivor mice were compared against published reference ranges (represented by the shaded regions of Figure 6A-F).[11, 15] All treatment groups had decreased cholesterol and glucose compared to normal reference ranges. Hypocholesterolemia and hypoglycemia may indicate mice had reduced nutrient intake or malabsorption. Potassium was elevated in all treatment groups and could be attributed to muscle damage given the elevations in creatine kinase in some of the mice from all three treatment groups. D-Doxo mice were also hypocalcemic; reduced calcium can be from increased renal or gastrointestinal loss or reduced calcium intake. Three D-Saline mice had mild hypophosphatemia that might have been a result of hemolysis during serum preparation.

Histologic changes were mild and were similar between treatment groups regardless of early mortality or survival. Lesions in the heart were focal to multifocal and had heart-to-heart variation by location of vacuolation, fibrosis, and periostin stain uptake. A study reported that

intracytoplasmic vacuolization was detected at higher rates 1 week after mice were administered a cumulative 25 mg/kg Doxo dose compared to 6 weeks after completion.[28] D-Doxo early mortality mice in this study were euthanized mostly during the second exposure period with no increased elevation in vacuolation compared to survivor mice.

Mouse strain differences might account for the discrepancy in findings. C57BL/6 mice have several published assessments of cardiotoxicity with Doxo administration.[14, 20, 21, 29] Not all studies distinguish what specific C57BL/6 substrain was used. A recent study demonstrated echocardiographic changes in C57BL/6N male mice after chronic administration of Doxo for a total of 30 mg/kg.[2] C57BL/6J and C57BL/6N mice have published differences in phenotype, such as immunity and metabolism, which could affect Doxo tolerance.[14] Systolic arterial pressure was found to be higher in C57BL/6J mice, heart rate was higher in C57BL/6N mice, and while under anesthesia the heart rate of C57BL/6N mice became significantly lower than C57BL/6J.

This study demonstrates the importance of distinguishing between acute and chronic toxic effects of Doxo. Doxo doses over a given duration might be high enough to cause acute toxicity in the mice and result in mortality. The mild histologic changes to the hearts in this study suggests severe chronic cardiotoxic Doxo effects that result in mortality may take longer to develop than 24-weeks in C57BL/6J mice. Frequency of dosing also determines the development of cardiotoxicity; higher refracted doses in a shorter amount of time leads to more severe lesions compared to a similar cumulative dose over a longer course of time.[23]

Though severe echo changes were not readily detected by fractional shortening and ejection fraction in this study, an alternative echo measurement to consider is strain. Strain can detect subtle cardiac changes in mice.[20] Magnetic resonance imaging is the gold standard for antemortem cardiac evaluation and can pick up even more subtle signs of cardiac dysfunction as well. Establishing defined and clinically relevant markers of cardiotoxicity in these mouse models would help allow Doxo cardiotoxicity studies to be comparable. For future studies involving Doxo, whole heart scans would allow for transparent evaluation of histology as cardiomyopathy appears to have a focal to multifocal distribution in mild cases.

Demonstrating that Acla will not exasperate Doxo associated cardiotoxicity in other populations would be essential to moving the study forward. Male mice were selected due to reports of increased sensitivity to Doxo compared to females, however in future directions this model should examine female mice and different age groups. Young and aged human patients have increased sensitivity to anthracycline toxicity. Mice were selected at 8-weeks of age to mimic cardiotoxicity in a developing young adult; mice hearts continue to grow until about 9-10 weeks of age.[16] Mice exposed to Doxo starting at 4-weeks-old can be used to explore a pediatric model.[26]

In conclusion, the results indicate Acla does not further potentiate Doxo cardiotoxicity. Acla was shown to be the favorable anthracycline of choice to administer after mice had received initial toxic dosages of Doxo. The cause of D-Doxo early mortality mice cannot be definitively attributed to cardiotoxicity based on echo, hematologic, and histologic assessment. Hematologic

analysis suggests early mortality mice presented with an inflammatory or stress leukogram, which could have a role in affecting survival.

FIGURES AND TABLES

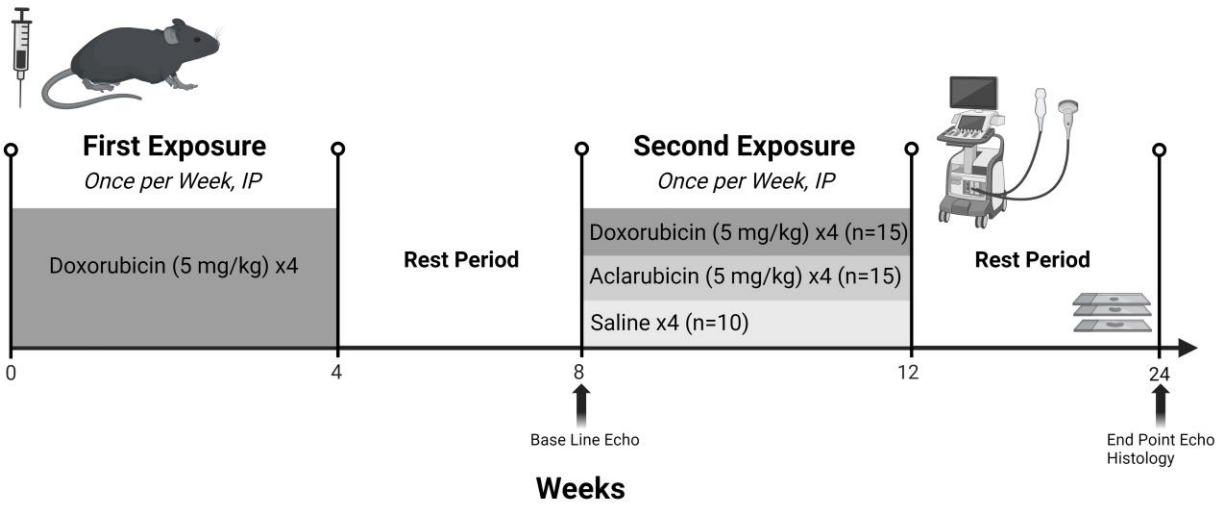

Figure 1. Study Design. Mice received IP injected Doxo (5 mg/kg) once a week for 4 weeks (total 20 mg/kg dose) in the first exposure period and are then rested for 4 weeks. Echocardiographic measurements are obtained at week 8. During the second exposure mice were administered treatment for an additional 4 weeks by their respective group: Doxo 5 mg/kg IP weekly, Acla 5 mg/kg IP weekly, or equivalent volume saline IP weekly (additional total 20 mg/kg dose of Anthracycline). Mice are rested for 12 weeks and at end-point mice receive a final echocardiograph and are euthanized for histologic evaluation.

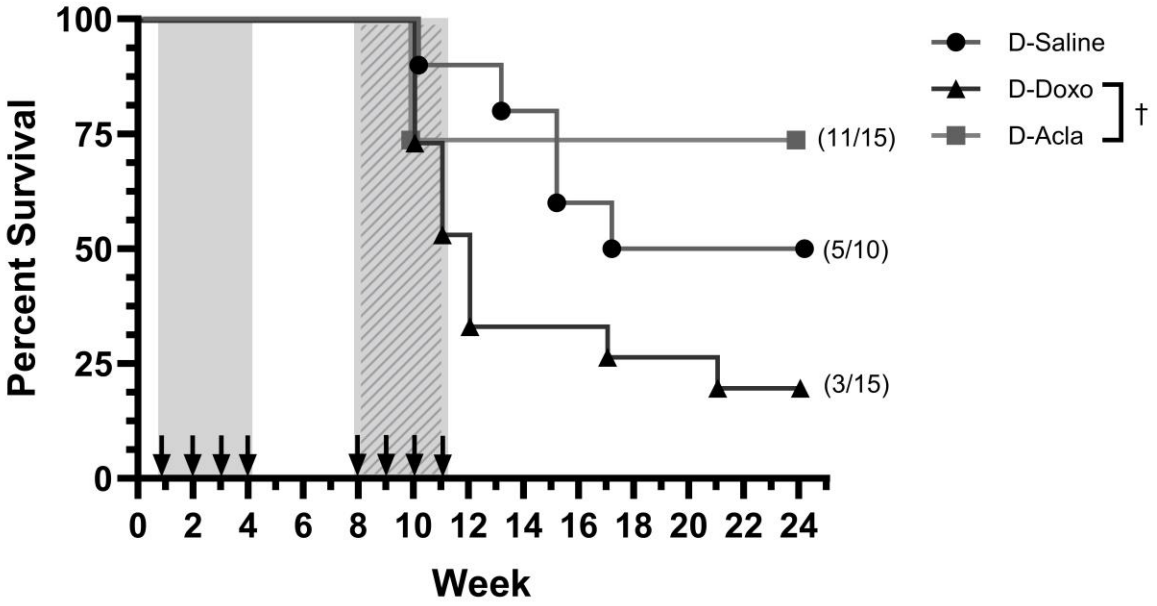

Figure 2. Survival Curve Log Rank (Mantel-Cox) test (†, $P \leq 0.01$). The grey box indicates the first exposure period, the shaded box represents the second exposure period, and the arrows represent weekly dosing.

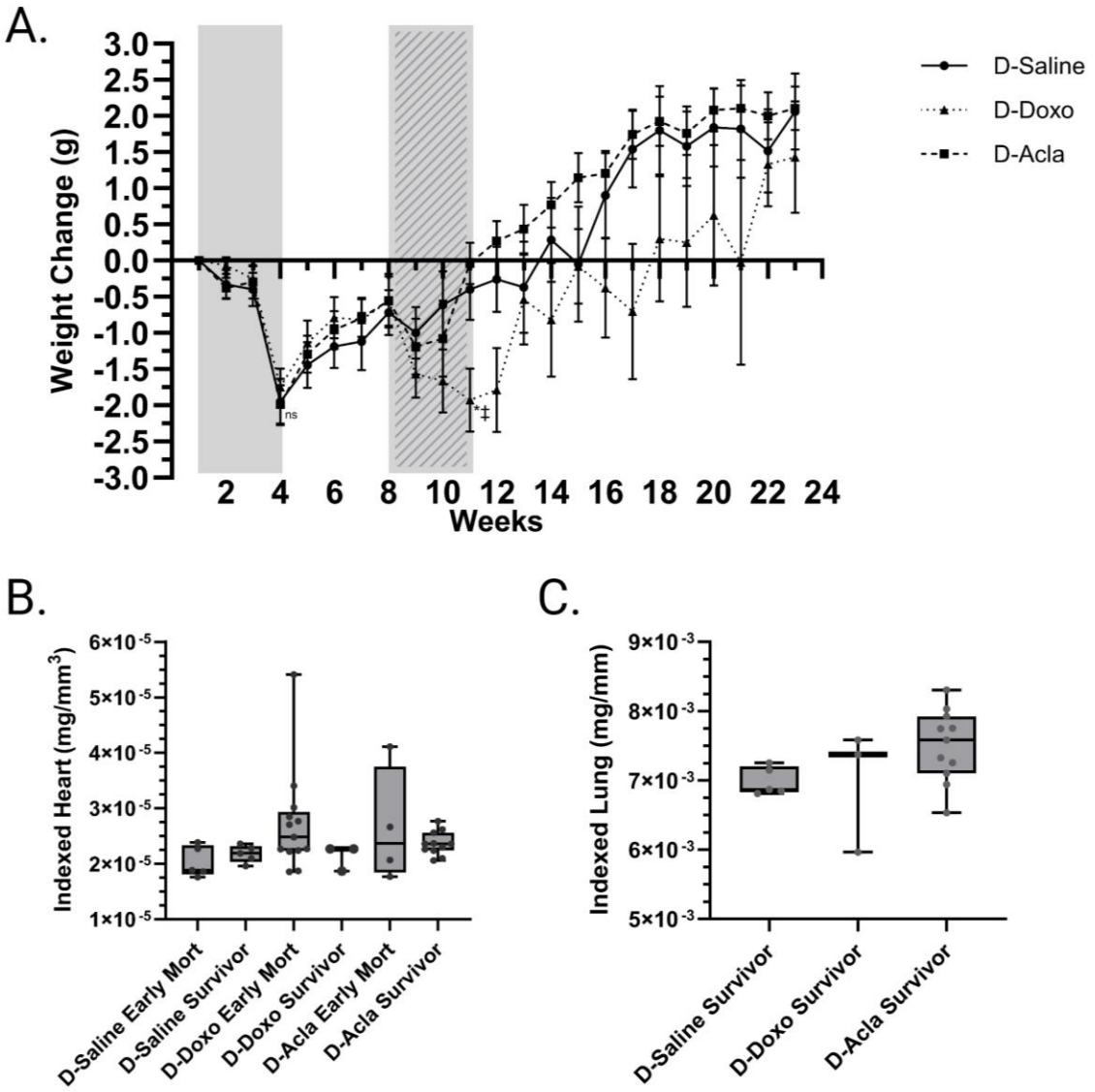

Figure 3. (A) Average weekly baseline weight change (mean ± SEM). Two tailed t-test performed at the end of exposure periods week 4 and week 11. Week 4: ns, no significant difference between any treatment groups. Week 11: *, $P \leq 0.05$ significant difference between D-Saline and D-Doxo; ‡, $P \leq 0.001$ significant difference between D-Acla and D-Doxo. Box plots: (B) Indexed heart weights for all treatments. (C) Indexed lung weights for all survivor treatments.

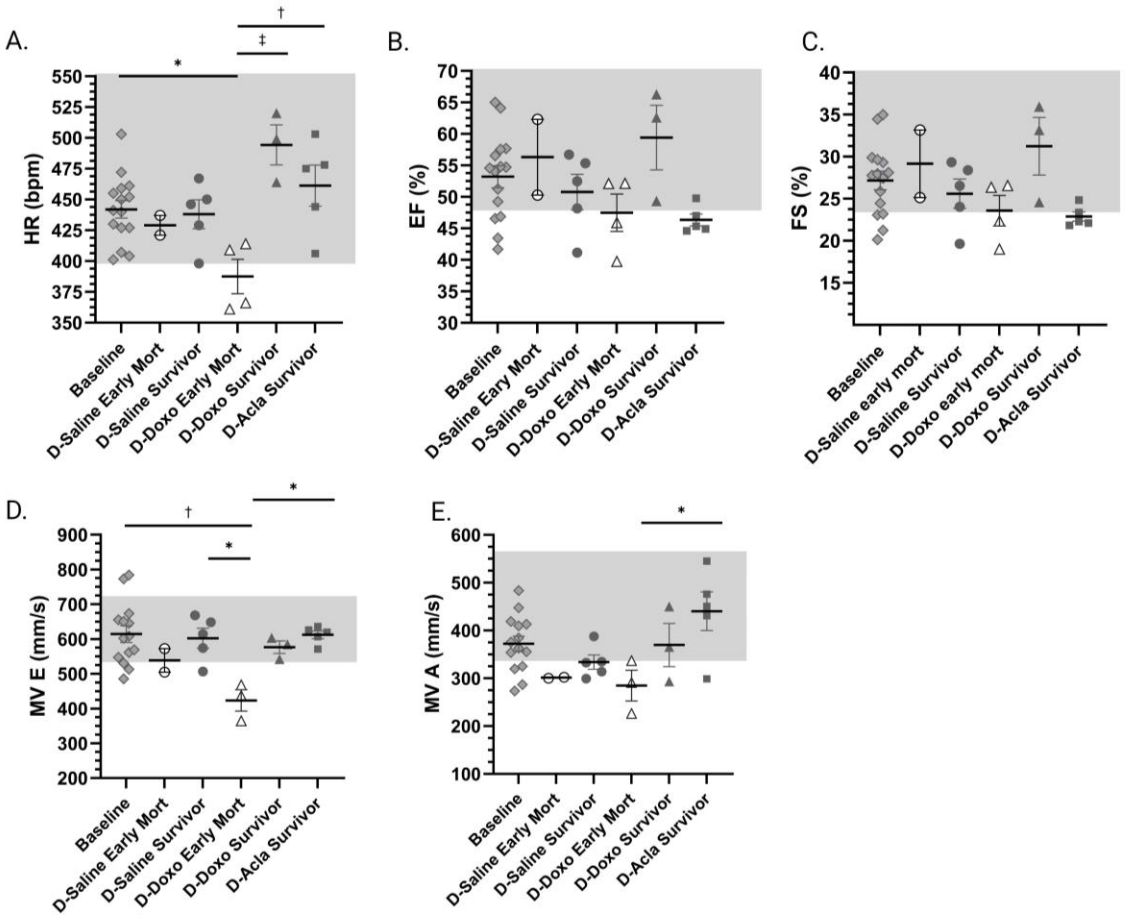

Figure 4. Scatter plots of echocardiographic measurements by early mortality and survivor treatment groups (Mean ± SEM; *, $P \leq 0.05$; †, $P \leq 0.01$; ‡, $P \leq 0.001$). (A) HR, Heart rate (B) EF, Ejection fraction (C) FS, Fractional shortening (D) MV E, Early diastolic trans-mitral flow velocity (E) MV A: late diastolic trans-mitral flow velocity.

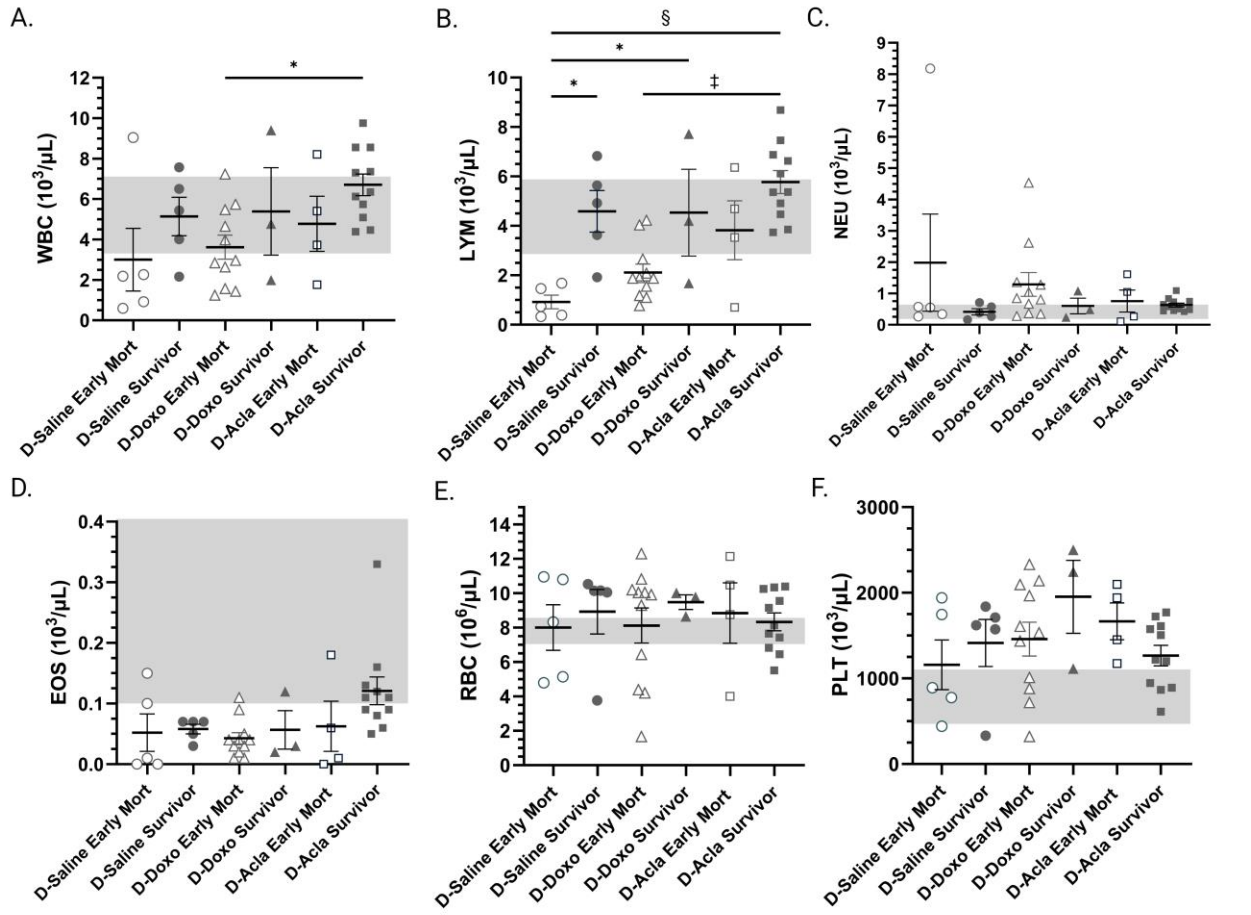

Figure 5. Scatter plots of complete blood count measurements by early mortality and survivor treatments (Mean ± SEM; *, $P \leq 0.05$; ‡, $P \leq 0.001$; §, $P \leq 0.0001$). Grey area represents published C57BL/6J expected references for experimentally naïve mice. [1] (A) White blood cell (B) Lymphocytes (C) Neutrophils (D) Eosinophils (E) Red blood cells (F) Platelets

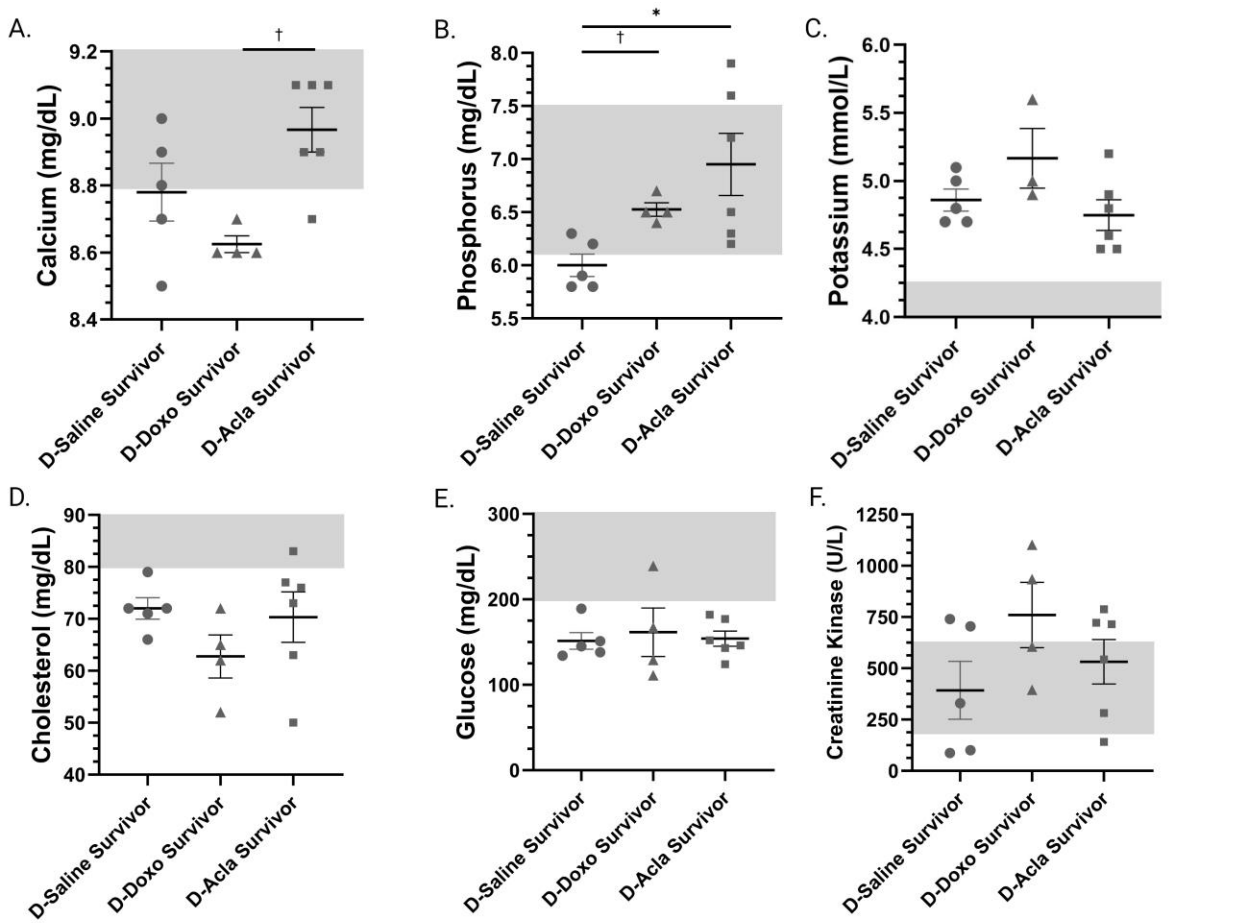

Figure 6. Scatter plots of serum chemistries in survivor mice only (Mean ± SEM; *, $P \leq 0.05$; †, $P \leq 0.01$). Grey area represents published expected reference ranges for experimentally naïve, adult, male, C57BL/6J mice. [15, 22] (A) Calcium (B) Phosphorous (C)Potassium (D) Cholesterol (E) Glucose (F) Creatinine kinase

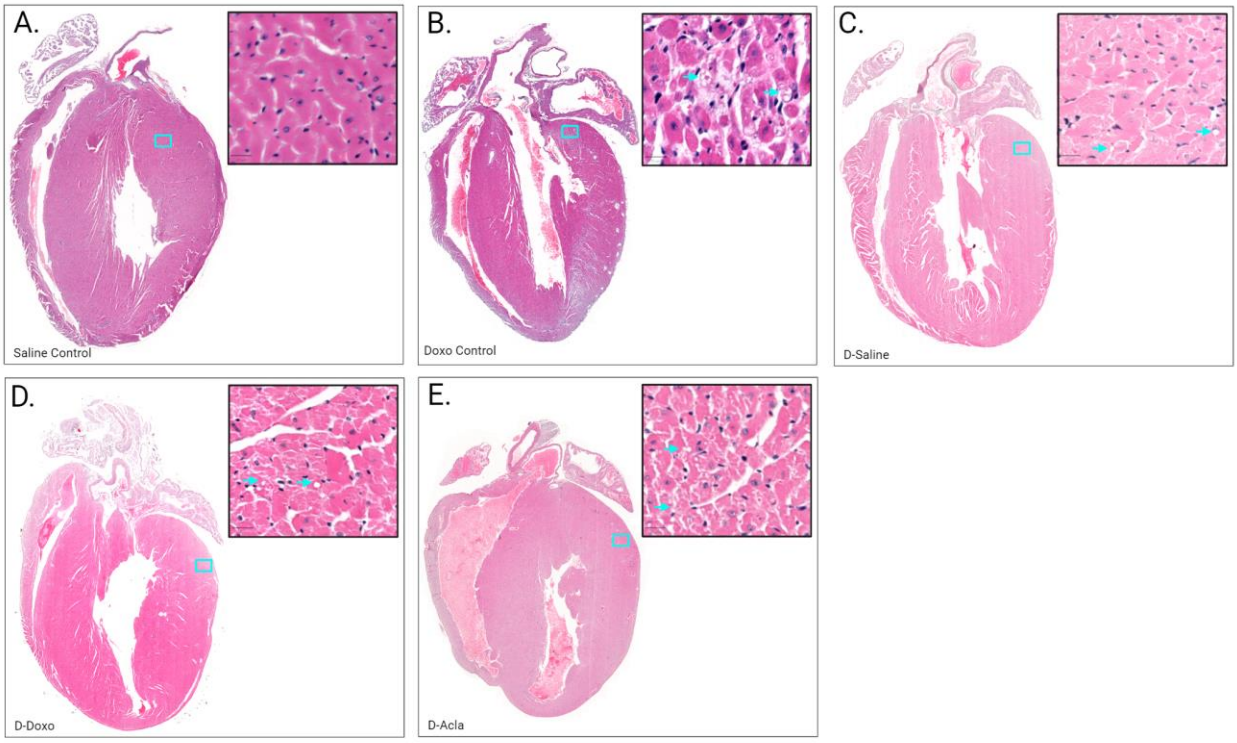

Figure 7. H&E whole heart scans with x12 magnification of blue boxed region. Blue arrow indicates areas of vacuolization. (A) Saline control from 18-week-old mouse that received IP injections of saline once a week for 6 weeks and rested for 12 weeks (B) Doxo control heart from 18-week-old mouse that received IP injections of Doxo 5 mg/kg/week x 6 and rested for 12 weeks (C) D-Saline (D) D-Doxo (E) D-Acla.

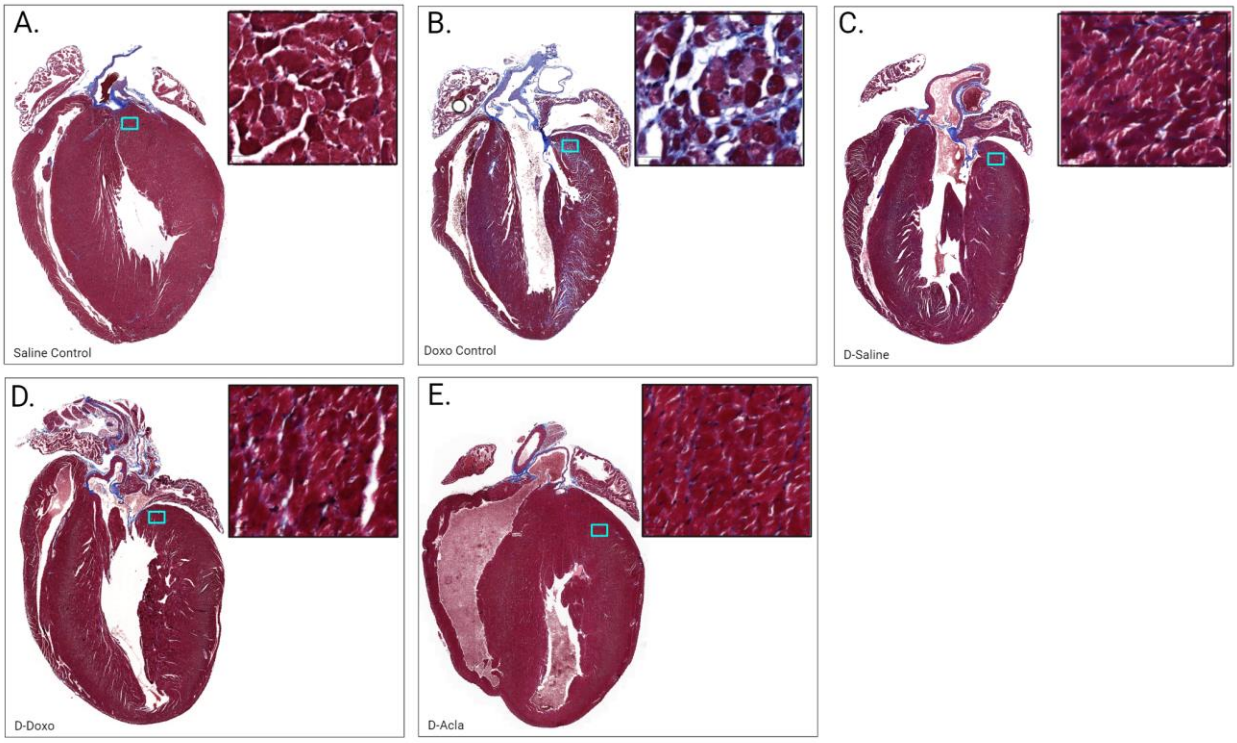

Figure 8. Trichrome whole heart scans with x12 magnification of blue boxed region. Blue staining indicates collagen. (A) Saline control from 18-week-old mouse that received IP injections of saline once a week for 6 weeks and rested for 12 weeks (B) Doxo control heart from 18-week-old mouse that received IP injections of Doxo 5 mg/kg/week x 6 and rested for 12 weeks (C) D-Saline (D) D-Doxo (E) D-Acla.

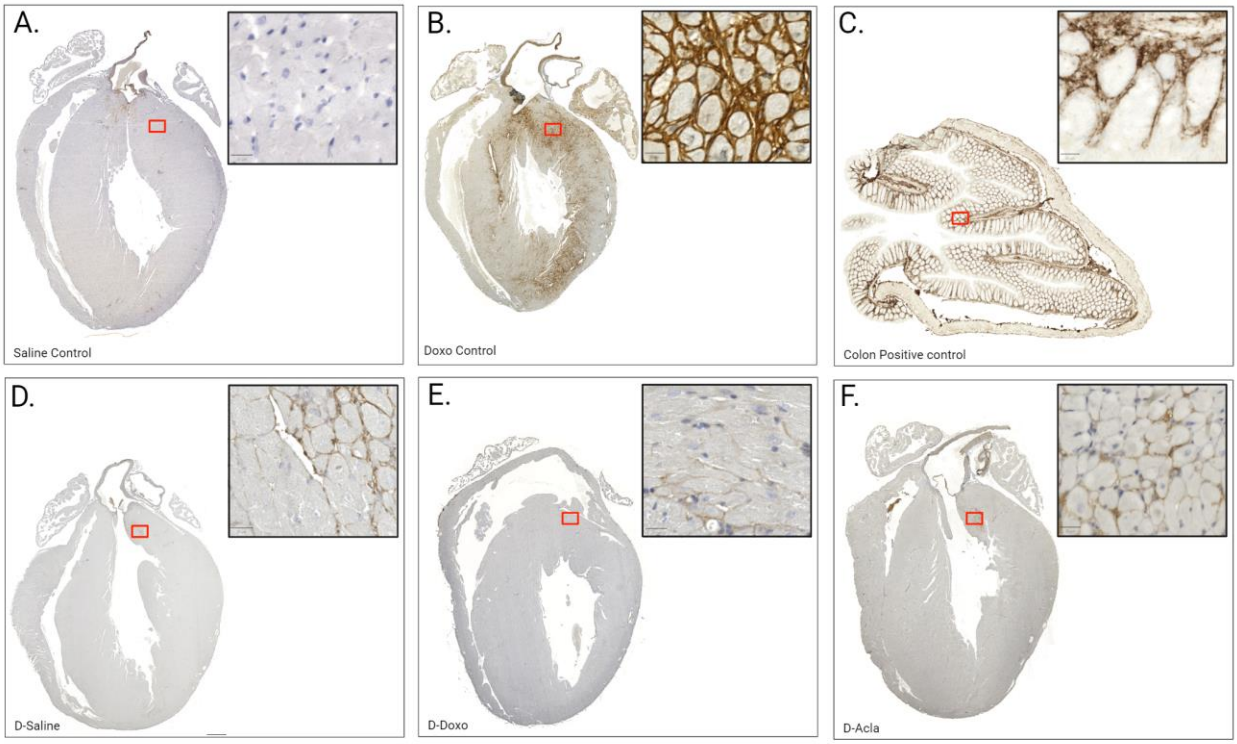

Figure 9. Immunohistochemistry staining for periostin whole heart scans with x12 magnification of blue boxed region. Brown staining indicates detection of periostin. (A) Saline control from 18-week-old mouse that received IP injections of saline once a week for 6 weeks and rested for 12 weeks (B) Doxo control heart from 18-week-old mouse that received IP injections of Doxo 5 mg/kg/week x 6 and rested for 12 weeks (C) D-Saline (D) D-Doxo (E) D-Acla.

Table 1. Histology scoring system for H&E, Trichrome, and Periostin.

H&E Vacuolation		Trichrome Fibrosis		Periostin IHC Staining	
0	None observed	**0**	None observed	**0**	None Observed
1	Rare	**1**	Rare	**1**	Minimal
2	<1/3 of area	**2**	<1/3 of area	**2**	<1/2 of area
3	1/3-2/3 of area	**3**	1/3-2/3 of area		
4	>2/3 of area	**4**	>2/3 of area		

Table 2. Scoring sample sheet for an individual heart.

Heart Area	Score		
	H&E	Trichrome	Periostin
Left Ventricle	0-4	0-4	0-2
Right Ventricle	0-4	0-4	0-2
Interventricular septum	0-4	0-4	0-2
Total Heart Score	0-12	0-12	0-6

Table 3. Median Survival of treatment groups.

Treatment Group	Median Survival (Week)
D-Saline (*n* = 10)	20.3
D-Doxo (*n* = 15)	12
D-Acla (*n* = 15)	Undefined

Table 4. Number of mice by treatment groups remaining after early mortalities[a]

Treatment Group	Experimental Week						
	10	11	12	13	15	17	21
D-Saline (*n* = 10)	9			8	6[b]	5[c]	
D-Doxo (*n* = 15)	11	8	5			4[d]	3
D-Acla (*n* = 15)	11						

[a]All euthanized mice lost greater than 15% of body weight
[b]Two mice presented hunched or minimal to moderately lethargic
[c]One mouse presented hunched with mild to moderate pale paws
[d]One mouse presented moribund with mild pale paws

Table 5. Histology total heart scores H&E, trichrome, and periostin by treatment groups (Mean ± SD). ns, no significant differences.

Treatment Group[a]	H&E Score	P value		
		S-D	S-A	D-A
D-Saline (*n* = 4)	7.0 ± 1.4	ns	ns	ns
D-Doxo (*n* = 9)	7.55 ± 2.45			
D-Acla (*n* = 3)	8.6 ± 1.33			
Treatment Group	Trichrome Score	P value		
		S-D	S-A	D-A
D-Saline (*n* = 9)	0.77 ± 0.46	ns	ns	ns
D-Doxo (*n* = 13)	0.46 ± 0.51			
D-Acla (*n* = 13)[b]	0.38 ± 0.50			
Treatment Group	Periostin Score	P value		
		S-D	S-A	D-A
D-Saline (*n* = 4)	0.66 ± 0.86	ns	ns	ns
D-Doxo (*n* = 13)	0.76 ± 0.83			
D-Acla (*n* = 14)	1.21 0 ± 0.80			

[a]H&E D-Saline (n= 9), D-Doxo (n= 13), D-Acla (n=13) slides all reviewed with few differences in severity; a subset of slides scored
[b]1 slide removed from evaluation due to staining artifact

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