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An established model of Doxorubicin-induced dilated cardiomyopathy in vivo

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Dear editor

With the invitation of Prof. Elizabeth Heppenheimer, we have submitted manuscript entitled “An established model of Doxorubicin-induced dilated cardiomyopathy *in vivo*” for possible publication in JoVE.

In our article, we successfully established a dilated cardiomyopathy model using Doxorubicin.

Best regards

TITLE:

A Doxorubicin-Induced Murine Model of Dilated Cardiomyopathy In Vivo

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KEYWORDS:

Doxorubicin, cardiotoxicity, dilated cardiomyopathy, heart failure, animal model, oncology-cardiology

SUMMARY:

Described is a protocol to establish a Doxorubicin-induced dilated cardiomyopathy (DCM) model in mice via long-term intraperitoneal injection of Doxorubicin.

ABSTRACT:

Dilated cardiomyopathy (DCM) refers to a spectrum of heterogeneous myocardial disorders characterized by ventricular dilation and depressed cardiac performance in the absence of hypertension, valvular, congenital, or ischemic heart diseases, and which may be related to infection, autoimmune or metabolic abnormalities, or family inheritance. It can progress into congestive heart failure with a poor prognosis. Doxorubicin (Dox) is widely employed as a chemotherapeutic drug, but its use is limited because it causes DCM-like changes of the myocardium. Its myocardial toxicity is attributed to oxidative stress, chronic inflammation, and cardiomyocyte apoptosis. A model of DCM exploiting these Dox-induced DCM symptoms has not been established.

INTRODUCTION:

One of the most common causes of heart failure, DCM is characterized by ventricular dilatation and decreased cardiac function and is the most common reason for heart transplantation worldwide[1]. In order to further investigate its pathogenesis and find effective treatments, access to mature animal models is especially important. The purpose of the described experiments is to establish a stable mouse model of DCM that resembles human DCM.

Due to the complex pathogenesis of DCM, there are many different methods to make corresponding animal models. Spontaneous DCM models[2] are relatively stable, but they are expensive and not easily available. Genetically modified animal models[3] are not well-established and require more experimental use. DCM animal models induced by viral infection[4] or autoimmune defects[5] are easy to obtain, but they are not wholly representative of DCM. Models associated with myocardial toxicity include alcohol-induced DCM models and Dox-induced DCM animal models.

The Dox-induced cardiomyopathy model is obtained by intraperitoneal injection of Dox[6]. The model exploits the most severe chronic side effect of Dox: After Dox exposure, patients develop late onset DCM symptoms with clinical uniformity[7]. Dox-induced oxidative stress[8] and mitochondrial damage[9], which lead to cardiomyocyte apoptosis, are symptoms in the pathogenesis of DCM. There are acute and chronic Dox treatment models: a single high dose of Dox (15 mg/kg) induces a short-term model for cardiomyopathy[10], while repetitive low-dose Dox injections (five weekly, 5 mg/kg) induce a long-term model for cardiomyopathy[11]. Based on the presented study, wild type mice intraperitoneally injected once a week for a month at a dose of 5 mg/kg display morphology and histology of the heart consistent with the characteristics of DCM by the end of the treatment, providing an ideal way to establish a DCM model.

PROTOCOL:

Animal experiments were approved by Institutional Animal Care and Use Committee (IACUC) of Nanjing Drum Tower Hospital.

1. Preparation of the reagents and animals

1.1. Dissolve doxorubicin hydrochloride (Pfizer, USA) in sterilized water. Vortex to obtain a Dox solution of 1 mg/mL and keep at 4 °C.

1.2. Use C57BL/6 mice (8–10 weeks old; 25–30 g weight). For this study, mice were purchased from the Model Animal Research Center of Nanjing University and kept in the animal room of Nanjing Drum Tower Hospital.

1.3. Pathogen-free mouse cages were maintained under a 12 h light/dark cycle at a constant temperature of 23 °C. All animals were fed on a normal chow diet and got food and water ad libitum.

2. Establishment of DCM animal model

2.1. Randomize mice into a normal (n = 5) group and Dox (n = 5) group.

2.2. Administer the Dox solution intraperitoneally at a dose of 5 mg/kg using a 1 mL sterilized syringe 1x a week for the Dox group. Treat the control mice in the same way with the same amount of saline solution.

2.3. Measure the body weight of the two groups weekly. Accordingly, adjust the injection dose based on body weight weekly for a total of 4 weeks, with a cumulative dose of 20 mg/kg (**Figure 1**).

NOTE: A time period of 4 weeks was chosen because echocardiography at 4 weeks showed a significant difference in cardiac function between the two groups.

3. Echocardiographic examination

3.1. At the end of the fourth week, conduct an echocardiographic examination of the mice.

3.2. Anesthetize the mice with 2% isoflurane intranasally. If the mice do not respond to a pinch of the skin with a toothed tweezer or stimulating the toes and tails, continue with the protocol.

3.3. Remove chest fur carefully with an electric shaver. Assess cardiac function in vivo using transthoracic echocardiography.

3.4. Perform the LV echocardiogram in both the parasternal long-axis and short-axis views at a frame rate of 233 Hz. End-systolic and end-diastolic dimensions were defined as the phases corresponding to the ECG T wave and to the R wave, respectively.

3.5. On the M-mode tracings, measure the average LV end-systolic diameter (LVIDd), LV end-diastolic diameter (LVIDs), interventricular septal thickness (IVS), and LV posterior wall thickness (LVPW) from 3–5 heart beats. Also calculate the ejection fraction (EF) and fraction shortening (FS) based on echocardiography.

4. Histological staining

4.1. After the echocardiographic analysis, sacrifice the mice by intraventricular injection of 10% potassium chloride.

4.2. Perfuse the heart with about 30 mL of saline after dissection until the liver and lung become pale.

4.3. Excise the heart and thoroughly wash it in phosphate buffer solution to extrude blood.

4.4. Fix the heart in 4% formalin at room temperature for 24 h and treat the tissue in a paraffin box so that the paraffin wax cools down and solidifies.

4.5. Cut the hearts into 5 µm thick slices for pathological staining.

4.6. Dewax and rehydrate sections containing papillary muscle.

4.6.1. Incubate slides at 55 °C for 30 min. Then, incubate in xylene 2x for 2 min each; 100% ethanol 2x for 2 min each; 95% ethanol 2x for 2 min each; 80% ethanol for 2 min; 75% ethanol for 2 min; and 50% ethanol for 2 min.

4.7. Stain using hematoxylin and eosin (H&E) as well as Masson's stain.

REPRESENTATIVE RESULTS:

Cardiac function

Dilated cardiomyopathy is characterized by progressive ventricular dilatation and contractile dysfunction. **Figure 2** shows representative echocardiographic images of the two groups. Dox-treated mice showed markedly reduced left ventricular ejection fraction and left ventricular fractional shortening (**Figure 3A, B**). The LV diameter also increased in both the diastolic and systolic phases (**Figure 3C, D**). This revealed that Dox-treated mice had an impaired cardiac function.

Histological staining

Pathological staining was performed to observe the pathological changes of saline-treated mice and Dox-treated mice. Myocardial fibers of the mice in the control group were neatly arranged, without infiltrating leukocytes. In the Dox group, myocardial myofibers were disordered and broken, and cardiomyocytes were smaller and thinner (**Figure 4A**). Masson staining showed that Dox-treated mice had more interstitial fibrosis (**Figure 4B**).

FIGURE AND TABLE LEGENDS:

Figure 1. Schematic diagram of a Dox-induced dilated cardiomyopathy.

Figure 2. The echocardiography of the two groups. (**A**) Control group; (**B**) Dox group. Left is the long-axis section and right is the short-axis section.

Figure 3. The cardiac function variables between groups. (**A**) EF; (**B**) FS; (**C**) LVEDd; (**D**) LVEDs. *P < 0.05, Student's *t*-test.

Figure 4. Histological staining. (**A**) H&E staining; (**B**) Masson staining. Left is the control group and right is the Dox group. The upper arrow shows that Dox-treated cardiomyocytes were smaller and thinner and the myofibers were disordered and broken. The lower arrow denotes the presence of interstitial fibrosis.

DISCUSSION:

Dox is a nonspecific periodic antitumor chemotherapy drug commonly used in clinical practice[12]. Its main side effect is cardiotoxicity, characterized by cardiomyopathy and subsequent heart failure[13]. The underlying mechanism includes damage of myocardial lipid peroxidation, inhibition of myocardial sarcoplasmic reticulum Ca^{2+}-ATPase activity, and

activation of the myocardial local renin-angiotensin system, resulting in increased AT II production and intracellular calcium overload[14]. Due to the lethal effect of a large dose of intraperitoneal injection of Dox, an alternative way to establish a reliable model was used in this protocol.

The DCM model established by this method is very similar to the pathogenesis of human DCM. In addition to heart failure, the most common complication of Dox, ascites were observed in DCM mice. Therefore, during model development, attention should be paid to sterile operation and timely replacement of syringes. In addition, for the Dox dose, two methods of administration were compared: a long course of treatment using a small dose, and short-term treatment with a high dose. The results show that continuous injection of Dox at 5 mg/kg for four weeks can result in an accurate model of DCM, lower mice mortality, and a shorter time for model establishment.

In summary, a stable, reliable, and economical mouse DCM animal model can be successfully established by intraperitoneal injection of Dox. This research provides an ideal model for studying DCM. It will contribute to reveal the pathogenesis of dilated cardiomyopathy or chronic heart failure and explore new drugs or treatments for future clinical applications.

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DISCLOSURES:
No conflicts of interest declared.

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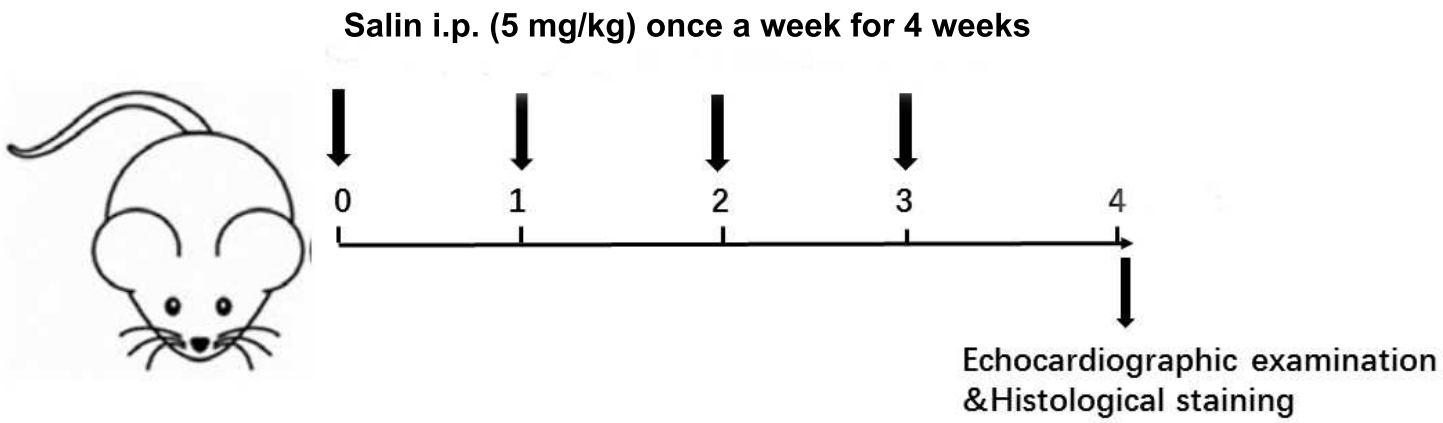
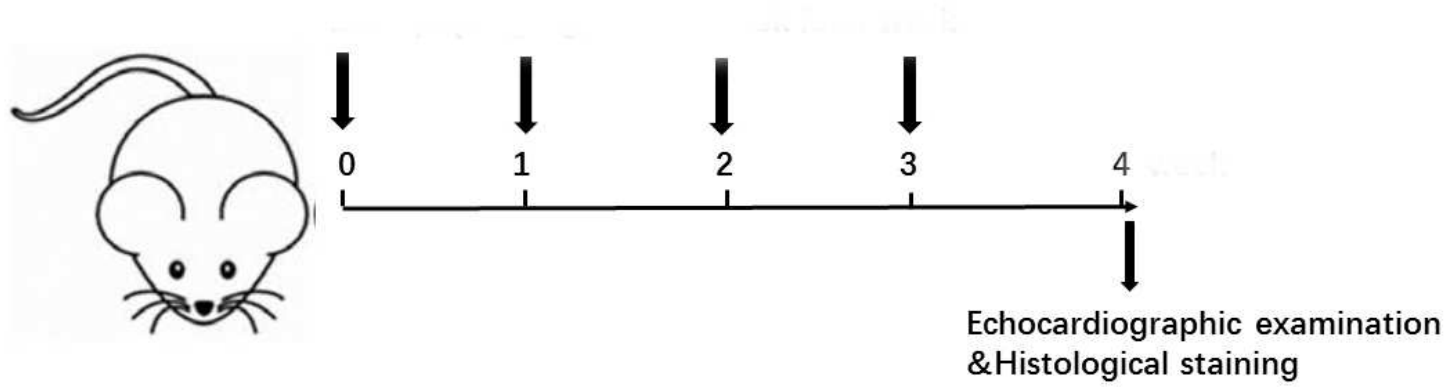
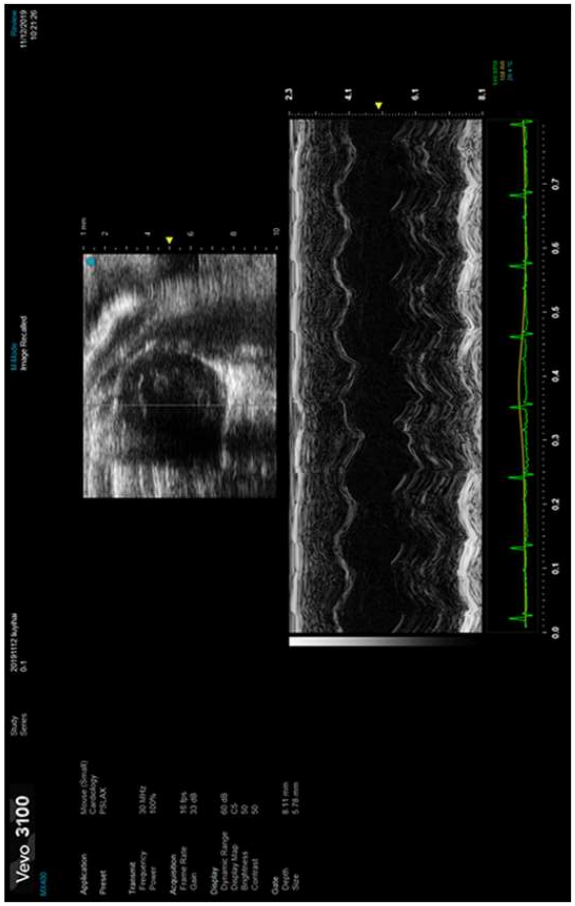
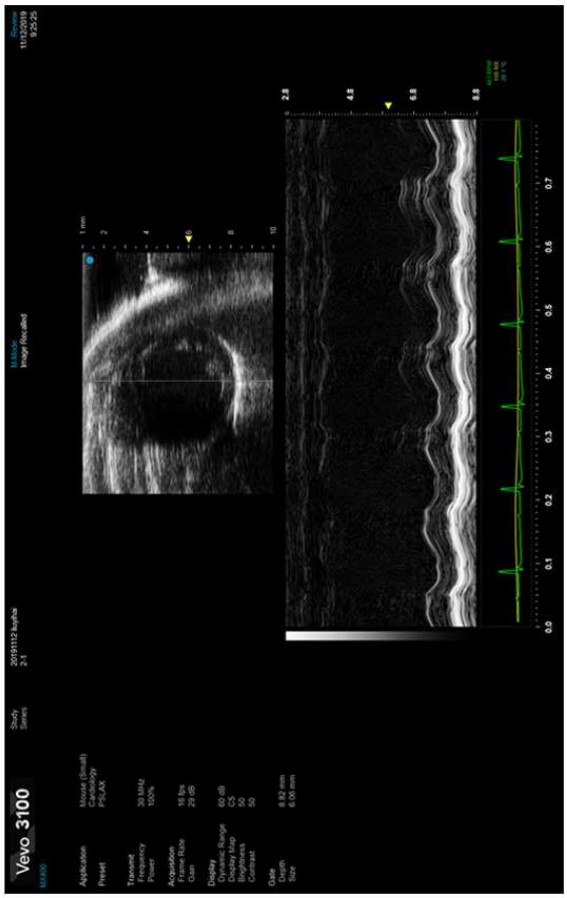
Control



Doxorubicin







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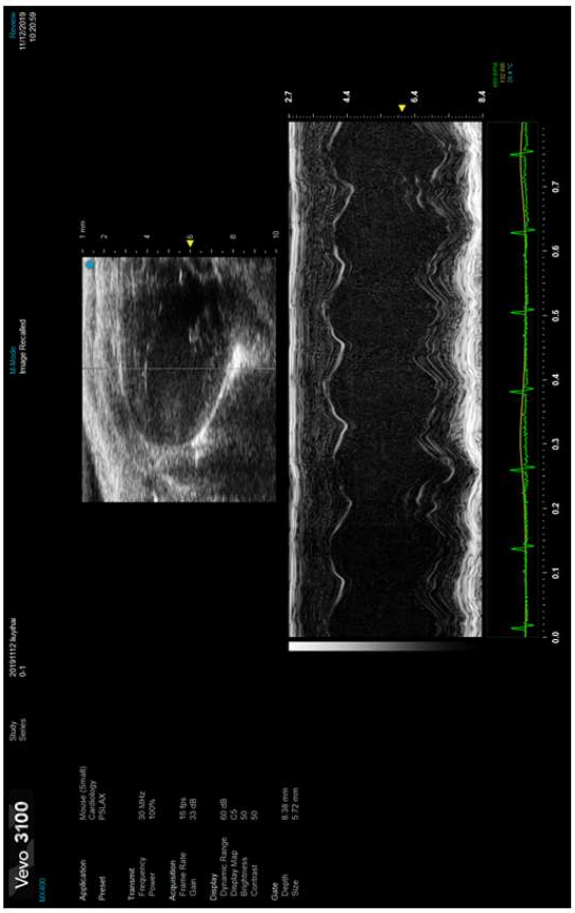

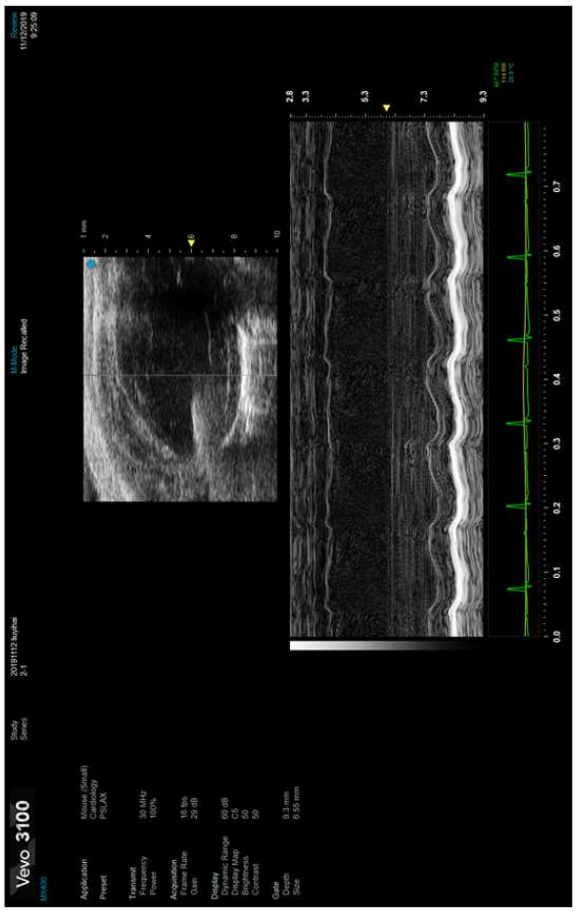

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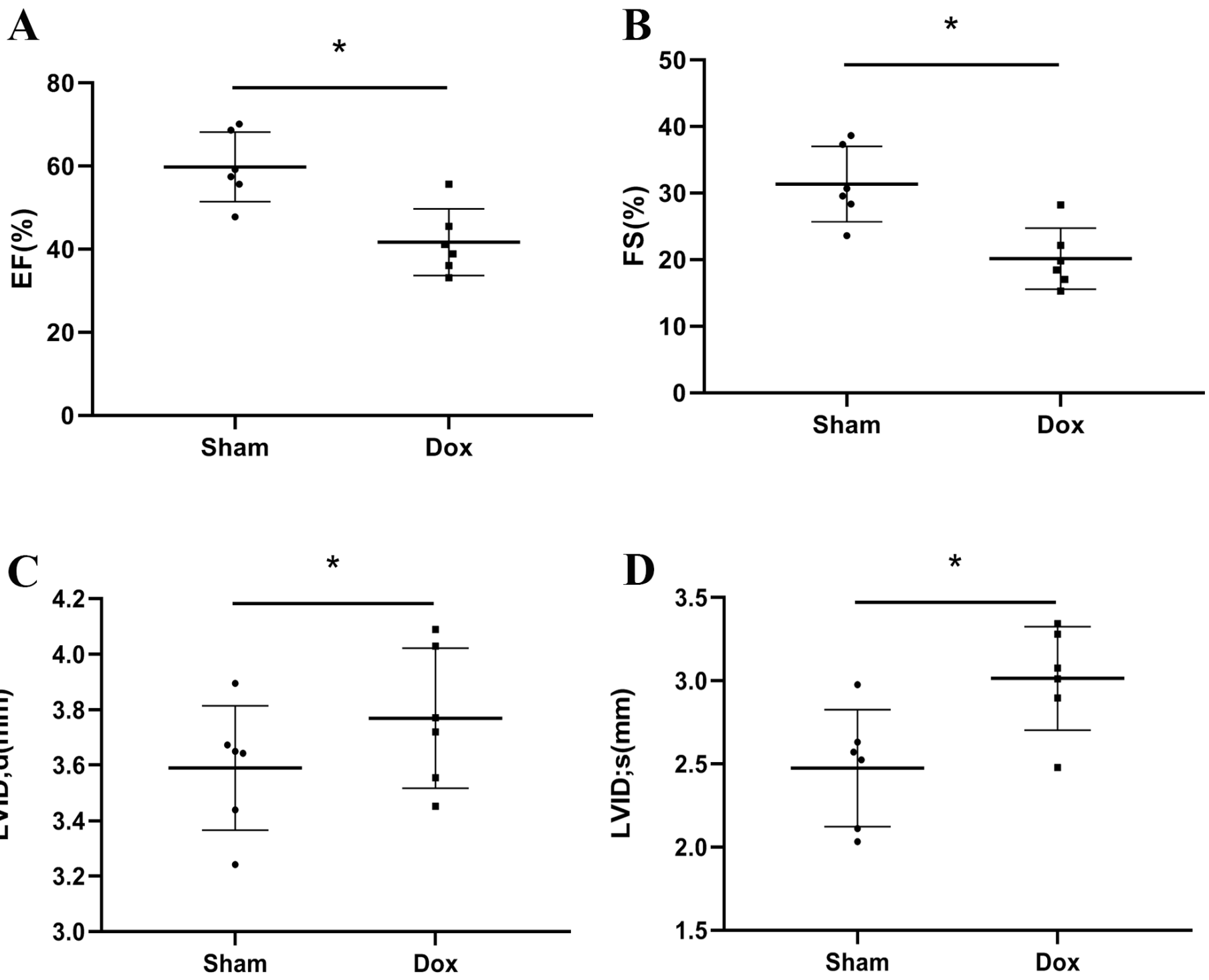

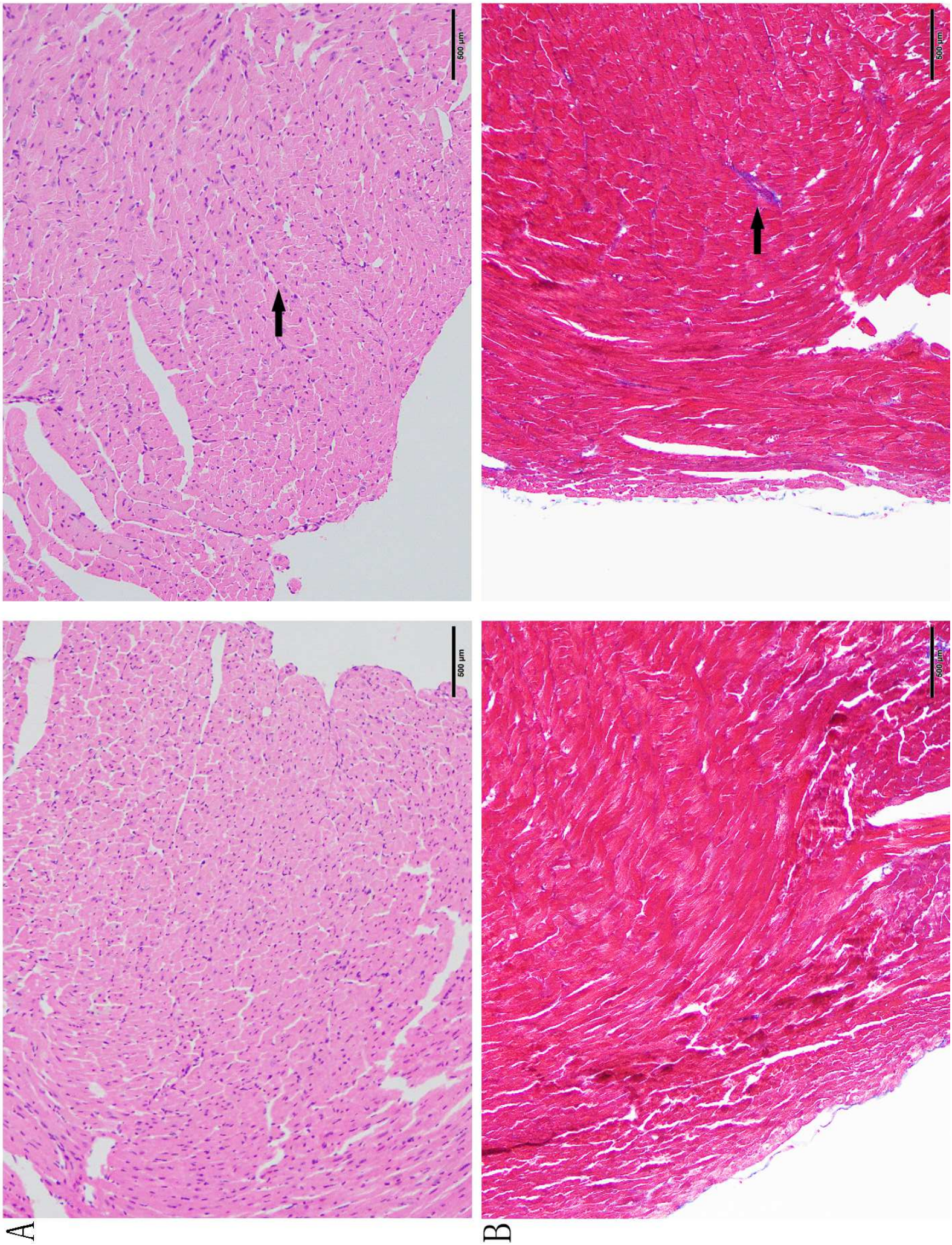

Name of Material/Equipment	Company
4% paraformaldehyde	servicebio
C57BL/6 mice	Model Animal Research Center of Nanjing University
Doxorubicin hydrochloride	Pfizer
echocardiography	Visualsonics
Hematoxylin and Eosin staining kit	Solarbio
Masson staining kit	Solarbio
phosphate buffer solution	Sigma
potassium chloride	Sigma
sterilized syringe	Millipore

Catalog Number	Comments/Description
CAS30525-89-4	
\	
CAS25316-40-9	
\	
G1120	
G1343	
P5368	
CAS7447-40-7	
SLGP033RB	

Editorial comments:

1. 2.3: Please provide an explanation for why 4 weeks was chosen in the manuscript itself.

First, this is a chronic model for weeks and the cumulative dose of > 15 mg/kg is appropriate based on previous work (PMID:1644973, PMID: 21330293). Besides, the echocardiography at 4 weeks showed the significant difference of cardiac function between two groups. So we chose 4 weeks in the manuscript itself.

2. 4.5-4.6: Do you perfuse the heart after dissection?

Yes, we perfused the heart with about 30 ml saline after dissection until the liver and lung became pale.

3. 4.5-4.6: How exactly do you dewax and hydrate? Which sections do you use, and how do you identify them?

The procedures were listed: Incubate slides, 55°C, 30 min; Xylenes, 2 times, 2 min, each; 100% EtOH, 2 times, 2 min, each; 95% EtOH, 2 times, 2 min, each; 80% EtOH, 2 min; 75% EtOH, 2 min; 50% EtOH, 2 min.

The 5μm-thickness transverse section was used, identified by the presence of papillary muscle.

4. Figure 2: What is the different between the images on the left and on the right? It may also be useful to label systole and diastole.

Left is long-axis section and right is short-axis section. They are both in systole phase.

5. Figure 3: What statistical test was used to obtain the p-value (e.g., t-test)?

The statistical test was student's t test.

6. Figure 4: Again, what are the images on the left and right? In general, this still could use more explanation; it is hard to see the differences that you are claiming (in particular, what exactly the arrows are supposed to be showing). More explicit demonstrations of the shape of the myofibers, etc. would be best.

The left is sham group while the right is Dox group. In the right image of A, arrow showed that the myofibers were twisted, broken and thin compared with sham group. In the right image of B, arrow showed the presence of interstitial fibrosis. Maybe we can perform the histological staining again for showing the difference, but it will take a long time.

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12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.