Original Article

# Efficacy of Different Cumulative Doses of Doxorubicin in the Induction of a Dilated Cardiomyopathy Model in Rats

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## Abstract

**Background:** **Doxorubicin (DOXO) has been used to induce dilated cardiomyopathy (DCM) in experimental models.**

**Objective:** **To analyze cardiac changes after DOXO infusion and define the most effective protocol to reproduce an experimental model of DCM.**

**Methods:** **Male Wistar rats were divided into 4 groups and received increasing cumulative doses of DOXO (at a rate of 2 mg/kg/week) or saline solution: the control group (CTR) received saline solution, Group D-8 received a total infusion of 8 mg/kg, Group D-12 received 12 mg/kg, and Group D-16 received 6 mg/kg. All animals underwent echocardiography at baseline and after the end of infusion. The animals were then euthanized and cardiac tissue was collected for histological analysis.**

**Results:** **Mortality rates were 20% (D-8), 30% (D-12), and 67.6% (D-16). The 8 mg/kg dose was not associated with a significant reduction in left ventricular ejection fraction (LVEF) or an increase in left ventricular end-diastolic diameter (LVEDD). There was significant LVEF impairment with 12 mg/kg and 16 mg/kg doses compared to the control (68.3±5% vs 58.4±9%, $p < 0.01$, for CTR-12 vs D-12; and 66.0±6% vs 47.6±15%, $p < 0.01$, for CTR-16 vs D-16). Histological analyses revealed a greater percentage of fibrosis in D-12 (10.6±3.3%) and D-16 (9.8±2.3%) compared to CTR (2.3±1.0%), $p < 0.001$.**

**Conclusions:** **The DOXO dose of 16 mg/kg was associated with severe cardiac changes and high mortality. Thus, we propose a DOXO dose of 12 mg/kg as the most appropriate and effective for inducing DCM with an acceptable mortality rate.**

**Keywords:** **Doxorubicin; Cardiotoxicity; Heart Failure; Left Ventricular Dysfunction; Dilated Cardiomyopathy.**

## Introduction

Animal models of cardiovascular disease are crucial for investigating pathophysiological mechanisms and testing new therapies.[1,2] Due to the high prevalence and clinical relevance of heart failure (HF) syndrome, several studies have described different models for analyzing this condition.[3-5] In this context, experimental HF models should mimic the major pathophysiological and morphological changes detected in humans, including cardiac remodeling, reduced ventricular function, hemodynamic changes such as reduced cardiac output and increased systemic vascular resistance, and histopathological changes. Over the last decades, several experimental models of acute and chronic HF with reduced ejection fraction have been developed to reproduce different aspects of dilated cardiomyopathy, a condition that can be induced by different events such as volume overload,[6] pressure overload by aortic constriction,[7] induction of arterial hypertension,[8] tachycardiomyopathy,[9,10] acute myocardial infarction,[11,12] and the use of cardiotoxic drugs such as propranolol, imipramine, and doxorubicin.[13,14]

Doxorubicin (DOXO), an anthracycline antineoplastic agent, is one of the drugs most frequently employed by investigators to induce dilated cardiomyopathy and HF.[15-21] DOXO is associated with dose-dependent cardiotoxicity, which may ultimately progress to HF. However, there is wide variation among protocols regarding the total cumulative dose of DOXO, the interval between doses, and the duration required to induce cardiopathy. In addition, the efficacy of these models in producing structural and functional changes that are consistent with those detected in human dilated cardiomyopathy while inducing acceptable mortality rates has not been clearly defined.

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To understand these findings in greater depth, the present study aims to investigate the morphological and functional cardiac changes induced by different cumulative doses of DOXO in rats in terms of the associated mortality rate and to define the most effective and high-yield induction protocol that would reproduce an experimental model of non-ischemic dilated cardiomyopathy.

## Methods

### Animals

Adult male rats with a mean body weight of 250 g were obtained from the Central Animal House of the Ribeirao Preto Medical School, Universidade de São Paulo (FMRP-USP). They were maintained in a climate controlled environment on a 12-h light/dark cycle with free access to water and standard chow. The number of animals allocated to each experimental group was based on previous studies and (an expectation of high mortality, especially in the groups that received DOXO infusion. This expectation was based on a series of prior reports in the literature on the use of DOXO to induce non-ischemic dilated cardiomyopathy, in which mortality was around 50 to 60%.[15,22] The Research Ethics Committee at FMRP approved all experimental procedures (protocol No. 041/2005).

### Chemical products

Adriablastin® RD (doxorubicin chloride) was purchased from Pfizer (Pharmacia, Milan, Italy), dissolved in saline solution (10 mg/100 mL), and administered by intravenous injection. In addition, intramuscular injections of ketamine hydrochloride (Vetbrands, Jacareí, SP, Brazil) and xylazine (Calier, Les Franqueses del Vallés, Barcelona, Spain) were used for anesthesia.

### Experimental protocol

A total of 60 animals were randomly divided into 3 experimental groups and 1 control group without specific criteria, receiving intravenous infusions with increasing cumulative doses at a rate of 2 mg/kg/week of DOXO or saline solution: Group D-8 (n = 20 animals) received a total infusion of 8 mg/kg over 4 weeks, D-12 (n = 30 animals) received a total infusion of 12 mg/kg for 6 weeks, and D-16 (n = 28 animals) received a total infusion of 16 mg/kg over 8 weeks. The control group (CTR) consisted of 8 animals matched for age that received 0.9% NaCl solution of the same volume as the DOXO infusion for 8 weeks.

All animals underwent, at baseline and 2 weeks after the end of infusion, an in vivo assessment of ventricular function by echocardiography. Then, they were euthanized for histological assessment and quantitative analysis of collagen areas.

The animals were kept in cages with members of the same group. All of them were submitted to the same stress conditions and order of measurements to minimize potential confounders. All procedures were performed under anesthesia to reduce stress and pain. There was no restriction on feed and water.

### Echocardiographic assessment of ventricular remodeling and function

Cardiac function was evaluated at baseline and after DOXO treatment by 2D echocardiography, as previously described.[23]

After sedation with ketamine and xylazine (20 and 8 mg/kg), the echocardiogram was recorded using a Sonos 5500 Philips (Andover, MA, USA) high-resolution two-dimensional echocardiography system with a 15-MHz high-frequency linear transducer. Using the parasternal window to obtain long-axis and short-axis images of the left ventricle (LV) at the papillary level, M-mode images were used to measure the interventricular septum, LV posterior wall thickness, and LV end-diastolic (LVEDD) and end-systolic (LVESD) dimensions. The diastolic diameter of the LV was measured at the maximum ventricular diastolic dimension, and systolic LV dimension was obtained during the maximum inward motion of the septum and posterior wall.

LV ejection fraction (LVEF) was calculated by the two-dimensional method, in which a two-dimensional LV shortening area was measured from the apical, subcostal, and, particularly, short axis views. In addition, images of left ventricular endocardial areas in diastole and systole were digitalized and measured offline. The shortening area was determined by the formula: EF (2D) = (EDA-ESA)/EDA, in which EDA and ESA are the end-diastolic and end-systolic areas, respectively.

The images were recorded by an echocardiography technician experienced in laboratory work with small animals who was blinded to the groups during the offline analysis at the end of the study.

All measurements represented the mean of five consecutive cardiac cycles using the same transducer position and angle in the same image frame. The interval between two consecutive cardiac cycles was measured for calculating heart rate.

### Histopathology – harvesting and preparation of hearts

Six animals randomly chosen from each group were used for histopathological analysis. The hearts were rapidly removed, rinsed in ice-cold 0.9% saline solution, and fixed as a whole by immersion in phosphate-buffered 10% formalin for 24 hours at 4°C for histological study. Both ventricles from each heart were isolated and cut into two fragments by a midventricular coronal section. Each block was serially cut in the same direction at a thickness of 4–7 $\mu$m appropriate for each stain, and sections were stained with hematoxylin and eosin (H&E) and picrosirius red.

### Collagen quantification

The sections stained with picrosirius red were used to quantify the interstitial collagen volume fraction using Leica Qwin Software V 3.2.0 (Leica Imaging Systems Ltd., Cambridge, UK) together with a Leica DMR microscope (Leica Microsystems Wetzlar GmbH, Wetzlar, Switzerland), video camera (Leica DC300F, Leica Microsystems AG, Heerbrugg, Switzerland), and an online computer. Twenty

high-magnification fields (×400) of the LV free wall were randomly selected for each animal, and interstitial collagen volume fraction values were expressed as percentages of the total LV area.

### Statistical analysis

Continuous variables are reported as means ± standard errors of the mean, and nominal variables are reported as absolute (n) and relative (%) frequencies. Data were analyzed using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA, USA). The Kolmogorov-Smirnov test was used to verify the Gaussian distribution of the variables. Student's t-test was used to compare the results between experimental and control groups. The Kruskal-Wallis nonparametric test, followed by Dunn's post-test, were used to evaluate the differences between the mean scores obtained at fibrosis quantification. Fischer's exact test was used to compare frequency distributions. The level of significance was set at $p < 0.05$, two-tailed in all analyses.

## Results

### Mortality

Two weeks after the end of the drug infusion period, in group D-8, four of the 20 animals that had started the experiment died (20%). In group D-12, mortality was 30% (9/30 animals), whereas mortality was extremely elevated in D-16: 67.6% (19/28 animals). No animals in the CTR group died (0%). A significant difference in mortality was detected only between group D-16 and its respective CTR group ($p < 0.001$), Figure 1.

### Body weight

A significant reduction in body weight (compared to the respective controls) occurred in animals in group D-8 (368±32 g vs 444±19 g, $p < 0.01$), group D-12 (366±30 g vs 505±23 g, $p < 0.0001$), and group D-16 (331±21 g vs 534±29 g, $p < 0.0001$). This difference was progressively more evident with the increasing DOXO dose, since the control animals weighed, on average, 20%, 38%, and 61% more than animals in groups D-8, D-12, and D-16, respectively.

### Functional and structural LV evaluation by echocardiography

*Baseline evaluation:* Baseline echocardiography data are presented in Table 1. No significant difference in echocardiography parameters was detected at baseline between groups ($p > 0.05$) before DOXO administration.

*Evaluation after doxorubicin infusion:* The echocardiography data obtained after DOXO infusion are presented in Table 2.

LVESD was larger in group D-16 compared to the respective control: 5.1±0.8 mm vs 4.3±0.5 mm, $p < 0.05$. This variable presented no differences in the remaining experimental groups. The final LVESDs of animals in the D-8, D-12, and D-16 groups were similar to those of their controls, $p > 0.05$.

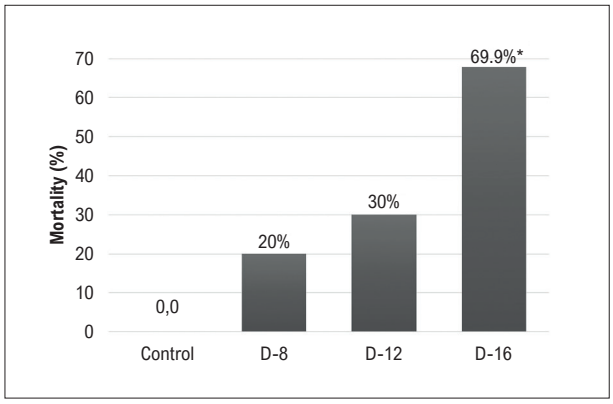


**Figure 1 –** *Mortality rate detected in the study groups (*p < 0.001 – Fisher's exact test).*

**Table 1 – Baseline echocardiography parameters assessed in all 86 studied animals**

| | CTR (n=8) | D-8 (n=20) | D-12 (n=30) | D-16 (n=28) |
|---|---|---|---|---|
| **Weight** (g) | 251.5±18.3 | 248.1±17.1 | 246.1±15.5 | 253.3±8.9 |
| **LVEDD** (mm) | 6.0±0.7 | 5.8±0.4 | 5.6±0.6 | 5.9±0.7 |
| **LVESD** (mm) | 3.1±0.8 | 2.8±0.5 | 2.7±0.4 | 3.0±1.1 |
| **SW thickness** (mm) | 1.5±0.2 | 1.6±0.2 | 1.6±0.1 | 1.4±0.2 |
| **PW thickness** (mm) | 1.5±0.2 | 1.6±0.3 | 1.6±0.2 | 1.5±0.2 |
| **LV mass** (g) | 1.1±0.1 | 1.0±0.1 | 1.0±0.1 | 1.1±0.1 |
| **2D LVEF** (%) | 72.2±9.9 | 72.7±4.9 | 73.5±7.5 | 72.2±6.5 |
| **Heart rate** (bpm) | 289±18.8 | 317±48.9 | 316±26.3 | 295±34.7 |

*CTR: control group; LVEDD: left ventricular end-diastolic diameter; LVESD: left ventricular end-systolic diameter; PW thickness: posterior wall thickness at maximum diastole; SW thickness: septal wall thickness at maximum diastole; 2D LVEF: ejection fraction determined by the method of LV fractional shortening. ANOVA followed by the Tukey-Kramer multiple comparisons test.*

LVEF results obtained by the two-dimensional method revealed that groups D-12 and D-16 exhibited a significant reduction of LV systolic function compared to their respective controls: 68.3±5% vs 58.4±9%, $p < 0.01$, for groups CTR-12 vs D-12; 66.0±6% vs 47.6±15%, $p < 0.01$, for group CTR-16 vs D-16. Thus, there was a 14.5% reduction in LVEF in group D-12 and a 27.9% reduction in group D-16. No decrease in LVEF was detected in group D-8, $p > 0.05$ (Figure 2).

The heart rate observed during echocardiography was significantly lower among D-16 animals (227±30 bpm) than among their controls (272±18 bpm), $p < 0.01$. There was no difference in this parameter for the remaining experimental groups.

**Table 2 – Mortality, weight, and echocardiographic parameters of the 58 surviving animals according to experimental group**

| | CTR-8 (n=8) | D-8 (n=16) | CTR-12 (n=8) | D-12 (n=21) | CTR-16 (n=8) | D-16 (n=13) |
|---|---|---|---|---|---|---|
| **Mortality** | 0% | 20% | 0% | 30% | 0% | 67.9% * |
| **Weight** (g) | 444±19 | 368±32 * | 505±23 | 366±30 * | 534±29 | 331±21* |
| **LVEDD** (mm) | 7.1±0.9 | 6.7±0.7 | 8.1±0.8 | 7.6±0.7 † | 8.1±0.4 | 7.7±0.8 † |
| **LVESD** (mm) | 3.5±0.8 | 3.6±0.5 | 4.2±1.1 | 4.5±0.7 † | 4.3±0.5 | 5.1±0.8 *†‡ |
| **SW thickness** (mm) | 1.5±0.2 | 1.5±0.1 | 1.7±0.2 | 1.6±0.2 | 1.6±0.1 | 1.5±0.1 |
| **PW thickness** (mm) | 1.5±0.2 | 1.6±0.3 | 1.6±0.3 | 1.5±0.1 | 1.5±0.1 | 1.4±0.2 † |
| **LV mass** (g) | 1.2±0.2 | 1.2±0.3 | 1.5±0.2 | 1.3±0.2 * | 1.3±0.1 | 1.2±0.1 |
| **2D LVEF** (%) | 70±8 | 66.7±5 | 68.3±5 | 58.4±9 *† | 66.0±6 | 47.6±15 †‡* |
| **Heart rate** (bpm) | 278±22 | 258±40 | 271±21 | 251±27 | 272±18 | 227±30 †‡* |

*CTR: control group; LVEDD: left ventricular end-diastolic diameter; LVESD: left ventricular end-systolic diameter; PW thickness: posterior wall thickness at maximum diastole; SW thickness: septal wall thickness at maximum diastole; 2D LVEF: ejection fraction determined by the method of LV fractional shortening. *p<0.05 compared to the control group according to age (unpaired t-test); †p<0.05 compared to group D-8 (ANOVA and Tukey post-test); ‡p < 0.05 compared to group D-12 (ANOVA and Tukey post-test).*

The LV mass estimated by echocardiography was significantly reduced in D-12 and D-16 animals compared to their controls, $p < 0.05$. No differences in estimated LV mass were observed between D-8 animals and their controls.

### Comparative analysis of different groups that received DOXO

A comparative analysis of the groups receiving different DOXO doses revealed a progressive decrease in LVEF (2D LVEF) between D-8 and D-12 ($p < 0.05$), D-8 and D-16 ($p < 0.001$), and D-12 and D-16 ($p < 0.05$) (Table 2).

### Quantitative analysis of histological changes by light microscopy

A histopathological analysis of the heart of control animals stained with H&E did not reveal any pathological changes, whereas the hearts of animals infused with DOXO showed myocyte injury with reduction and degeneration of myocardial fibers, a significant decrease of myofibrils with loss of myofibrillar organization, and periarteriolar fibrosis in the myocardium and interstices, associated with collagen deposition (Figure 3). The changes detected in group D-8 were less pronounced than in groups D-12 and D-16.

### Quantification of fibrosis

For a quantitative analysis of fibrosis, samples were stained with picrosirius red. A higher percentage of fibrosis was observed in groups D-8 (6.0±2.3%), D-12 (10.6±3.3%), and D-16 (9.8±2.3%) compared to the control (2.3±1.0%), $p < 0.001$. Additionally, groups D-12

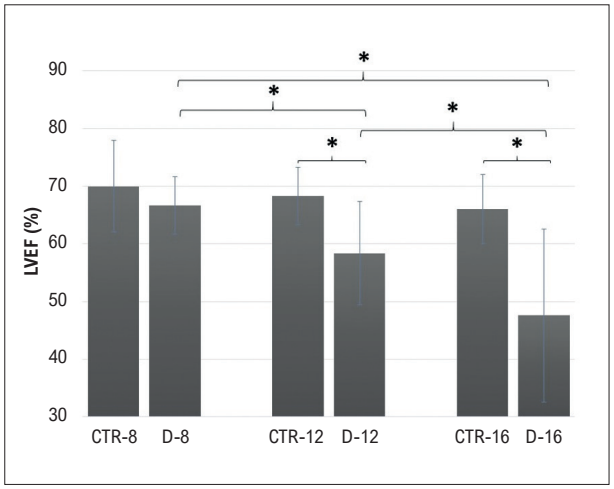


**Figure 2 –** *Mean left ventricular ejection fraction (LVEF) values obtained in each of the study groups. * p < 0.05 compared to the control group according to age (unpaired t-test) and compared between groups (ANOVA and Tukey post-test).*

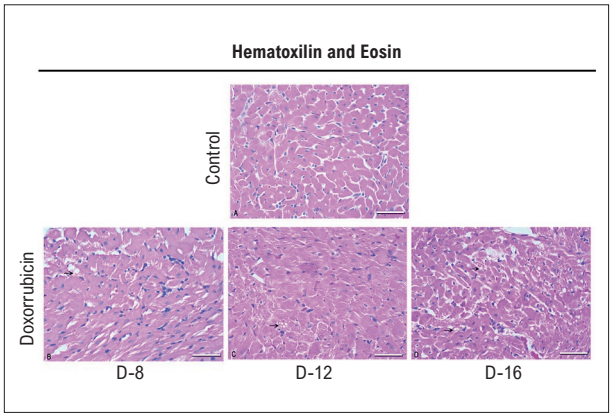


**Figure 3 –** *Representative histological sections stained with hematoxylin and eosin (H&E) from the control (CTR) and doxorubicin (DOXO) groups (D-8, D-12, and D-16). Myocardial fiber loss and degeneration are observed in animals after DOXO infusion (panels B, C, and D), with a significant reduction in myofibrils, edema, and vacuolar degeneration (marked by arrows). These changes are more pronounced with the increment of DOXO cumulative doses.*

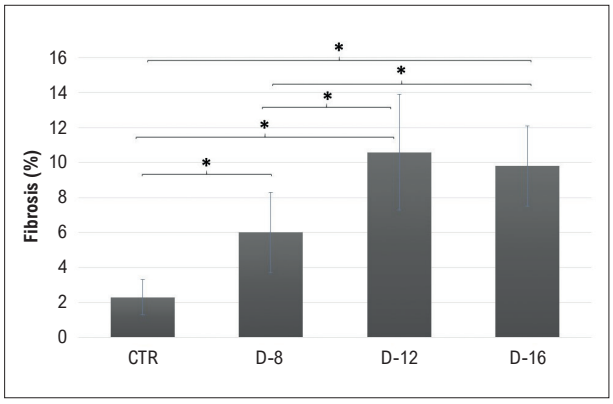


**Figure 4 –** *Mean fibrosis values obtained in the study groups. * p < 0.05 (ANOVA and Tukey post-test).*

and D-16 showed a larger area of fibrosis than group D-8, p < 0.001, but did not differ from one another (Figure 4).

## Discussion

In the present study, we investigated the effectiveness of different cumulative doses of DOXO in inducing functional and cardiac changes, balancing these results with mortality rates intrinsically associated with higher cumulative doses in order to define the dose of DOXO with the best efficacy. The main results showed a significant reduction of LV systolic function starting with a cumulative dose of 12 mg/kg of DOXO, with greater dysfunction occurring with the cumulative dose of 16 mg/kg and no significant decrease in LVEF with the 8 mg/kg dose. Additionally, we observed a significant increase in myocardial fibrosis compared to control in all 3 studied cumulative doses; the highest degree of fibrosis was observed in groups receiving 12 mg/kg and 16 mg/kg, which exhibited similar degrees of fibrosis. A progressive increase in mortality was associated with higher doses, with an excessive rate at the cumulative dose of 16 mg/kg (67.9%). Taken together, these results suggest that the dose of DOXO with the best efficacy for the induction of dilated cardiomyopathy in rats was 12 mg/kg.

### Mortality rates

The high mortality rate observed in the present study agrees with values reported in previous studies, ranging from 36 to 82%.[15,18,24,25] Previous studies have shown that variations in mortality rates are related to three main reasons: total dose administered, duration of the period of infusion, and time of observation. Mortality increases with higher doses, shorter periods of administration, and longer periods of observation.[15,24,26]

In addition to myocardial injury, high cumulative doses of DOXO produce renal, bone marrow, and gastrointestinal toxicity that may contribute to increased mortality rates due to the induction of hyperkalemia, hypervolemia, anemia, diarrhea, and malnutrition.[24,27] In addition to losing weight and muscle mass, the animals become weak and unable to feed properly, with a consequent progressive and generalized muscle weakness that may contribute to increased mortality. In the present study, animals in D-12 and D-16 showed significant differences in general aspect and weight compared to their respective controls, indirectly supporting the presence of these debilitating mechanisms. These changes have also been described by other authors.[15,22,24,27]

### In vivo structural and functional cardiac changes

In general, studies using DOXO were designed for investigating histopathological lesions[28] and studying the drug's cardiotoxicity,[29] mainly focusing on metabolic changes and oxidative stress[15,30-32] instead of assessing the degree of in vivo ventricular dysfunction or mortality. Thus, their objective was not to characterize a model of HF or dilated cardiomyopathy, and we found a wide variety of protocols in the literature. Our study intended to describe a protocol that would be adequate, effective, and of better yield, reproducing an experimental model of non-ischemic dilated cardiomyopathy based on in vivo structural and functional changes but with acceptable mortality rates.

We highlight that in this study, we chose the intravenous infusion of DOXO at increasing cumulative doses. In a previous study with rats, O'Connel et al.[33] compared 2 protocols of DOXO infusion, a short one and a prolonged one, in the induction of dilated cardiomyopathy. In this study, we observed that both protocols generated similar histological injuries, although only the prolonged infusion was associated with structural and functional changes similar to those detected in clinical dilated cardiomyopathy. These findings support the idea that a prolonged infusion time is more effective than a short infusion when the main outcome is the induction of structural and functional changes.[33]

In the present study, we observed a clear correlation between the cumulative dose of DOXO and the degree of ventricular dysfunction that occurs progressively from the cumulative dose of 12 mg/kg. Our results show that the animals receiving a cumulative dose of 16 mg/kg presented more marked functional and structural cardiac changes assessed in vivo by 2D echocardiography when compared to their respective control groups. These changes were mainly characterized by an increase in LVESD, a decrease in LVEF, and a reduction of estimated LV mass. However, the mortality rates for this group were excessive, since more than two-thirds of the animals did not survive up to two weeks after the induction of cardiomyopathy.

Conversely, the cumulative dose of 12 mg/kg was assciated with structural changes that were not as marked as those observed with the 16 mg/kg dose, but it enough to cause a significant reduction in LV ejection fraction and an increase in myocardial fibrosis. In addition, the mortality rate for this group (30%) was acceptable, leading us to conclude that this protocol might be adequate for the induction of ventricular dysfunction with DOXO in rats.

Our results corroborate the findings of previous studies suggesting that cumulative doses above 12 mg/kg are associated with increased mortality.[18,22,34] The results obtained by Spivak et al.[34] using a shorter DOXO dosing

time suggested that cumulative doses above 12 mg/kg are associated with a mortality rate of more than 40%, thus being inappropriate for in vivo research on HF.[34] Indeed, the study by Schwarz et al.[22] demonstrated that higher doses (such as 25 mg/kg over a period of 10 weeks) are associated with a higher degree of ventricular dysfunction but also with high mortality rates, reaching 52%.[22]

### Histopathological changes

In the present study, we observed clear morphological differences between groups. The injuries were typical from reports of DOXO-induced dilated cardiomyopathy in humans[35] and in other animal models,[36-38] ie, predominantly involving damage to the myocytes with loss and degeneration of myocardial fibers, a significant reduction in myofibrils, fibrosis and collagen deposition, in addition to edema and cardiomyocyte vacuolization, intracellular edema, and myofibril disorganization. The collagen deposition observed in our study was similar to that reported in previous studies using this experimental model.[23,39] The cumulative dose of DOXO associated with significant histological damage in our study was in agreement with reports that suggested a dose of 15 mg/kg as the most effective in inducing histopathological injury.[40]

It should be noted that even though ventricular dysfunction was more marked at the 16 mg/kg dose, the degree of tissue injury represented by fibrosis was similar in groups D-12 and D-16, suggesting a possible dissociation between these two parameters in this range of cumulative DOXO doses. These results support previous evidence obtained by our research group showing a relative dissociation between the degree of tissue injury and the intensity of left ventricular dysfunction with the use of different regimens of DOXO infusion.[33]

Our results show that the induction model using a total cumulative dose of DOXO of 16 mg/kg with 8 weekly injections of 2 mg/kg is the one that best leads to morphological and functional changes in the animals, but it involves high, ethically unacceptable mortality, which discourages the use of this protocol. In addition, the difficulty in maintaining a highly aggressive model of ventricular dysfunction for the minimum period of time required for evaluating a given therapeutic intervention is a relevant factor in the choice of the most efficient protocol.

## Conclusions

Our results indicate that the model with 6 weekly injections of 2 mg/kg of DOXO, with a cumulative dose of 12 mg/kg, presents the best efficacy for inducing dilated cardiomyopathy, resulting in significant left ventricular remodeling, systolic function impairment, and tissue injury associated with acceptable mortality rates.

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## Author Contributions

Conception and design of the research: Tanaka DM, O'Connell JL, Schmidt A, Simões MV; Acquisition of data: O'Connell JL, Fabricio CG, Romano MMD, Campos EC, Oliveira LFL, Carvalho EEV; Analysis and interpretation of the data: Tanaka DM, O'Connell JL, Fabricio CG, Romano MMD, Campos EC, Oliveira LFL, Schmidt A, Carvalho EEV, Simões MV; Statistical analysis: Tanaka DM, Simões MV; Obtaining financing: Simões MV; Writing of the manuscript: Tanaka DM, Campos EC; Critical revision of the manuscript for important intellectual content: Tanaka DM, O'Connell JL, Fabricio CG, Romano MMD, Oliveira LFL, Schmidt A, Carvalho EEV, Simões MV.

### Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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### Study Association

This article is part of the thesis of doctoral submitted by João Lucas O'Connell, from Universidade de São Paulo.

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