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Time course of cardiomyopathy induced by doxorubicin in rats

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ABSTRACT

Background: Doxorubicin (DOX)-related cardiotoxicity may expose cancer survivors to increased risk of cardiovascular morbidity and mortality. Here, we characterized the time course of DOX-induced cardiomyopathy in rats.
Methods: Sprague-Dawley male rats (12 wk old) received doxorubicin hydrochloride (1 mg/kg/d, *ip*) during 10 consecutive days and they were euthanized one (DOX1), two (DOX2) or four (DOX4) weeks after the last drug injection. Control group received NaCl 0.9% (*ip*). Hearts were mounted on a Langendorff perfusion system, left ventricle fragments were processed for microscopy and oxidative stress-related assays, and blood was collected for cardiac troponin I assay.
Results: All DOX-treated groups showed swollen and vacuolated cardiomyocytes with myofilaments disarray and mitochondrial damage. These changes were already evident after one week and became more pronounced after four weeks. Cardiac troponin I plasma levels were significantly increased in DOX1 and further increased in DOX4 compared to control group. Increased oxidative damage to lipids was observed in DOX1, and to proteins in DOX4. Glutathione peroxidase activity increased in DOX4. The morphological changes resulted in cardiac remodeling, including interstitial fibrosis, apoptosis and significant impairment of both contractile and relaxation function in DOX 4 compared to control group. Hearts from all animals displayed an early reduction in the responsiveness to norepinephrine.
Conclusions: These findings support the view that DOX cardiotoxicity occurs in a "continuum", and as the hypothesis of an irreversible cardiac injury is being challenged, understanding the progression of morphological and functional changes caused by DOX may allow proper timing of initiation of prophylactic treatment.

Introduction

An emerging field referred to as cardio-oncology has grown in recent years due to the increasing incidence of cardiovascular adverse effects induced by some types of cancer treatments [1]. Anthracyclines comprise the first class of compounds recognized to affect the heart, and they are still widely used in the treatment of solid tumors and hematological malignancies [2]. Doxorubicin is one of the most used anthracyclines, and its use is associated with a 48% incidence of heart failure after the use of a cumulative dose of 700 mg m^{-2} [3].

The mechanisms by which doxorubicin induces cardiotoxicity are still an open question, but two major hypotheses seem to prevail. The most accepted one is the reactive oxygen species (ROS) hypothesis, which states that doxorubicin undergoes redox recycling in complex I of the electron transport chain, resulting in massive ROS production, and subsequent damage to DNA, protein, and lipids that culminates in cellular dysfunction or death [4]. More recently, a second mechanism was postulated. Doxorubicin was shown to induce DNA double-strand breaks *via* topoisomerase 2β, resulting in activation of apoptotic pathways and cell death [5]. Regardless of the mechanism, doxorubicin leads to cardiac damage that has been traditionally accepted as a type I lesion, which is characterized by permanent and irreversible cardiac dysfunction [6]. However, this hypothesis is being challenged. Cardinale et al. [7] recently demonstrated that left

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ventricle dysfunction could be reversed in some patients when heart failure therapy (beta blockers and/or angiotensin converter enzyme inhibitors) was initiated soon after any evidence of cardiac dysfunction. When heart failure therapy was initiated up to two months after the end of chemotherapy, 64% of the patients presented full recovery of left ventricle function, particularly those without clinical symptoms when therapy was initiated.

Therefore, understanding the time course of cardiac insults after doxorubicin treatment may allow early recognition of the deleterious effects of this drug and, thus, a possibility to revert cardiac dysfunction. As such, the purpose of this study was to investigate the progression of both structural and functional cardiac changes induced by doxorubicin.

Material and methods

Animals and groups

The study protocol was approved by the local ethics committee of animal use (protocol number 023/2015). All experiments were performed according to National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). Twelve-week-old male Sprague Dawley rats weighing approximately 300 g were kept in ventilated cages, under controlled temperature and humidity. The animals were maintained on a 12-h light/dark cycle with food and water provided *ad libitum*. Doxorubicin (DOX) groups received intraperitoneal (*ip*) injections of doxorubicin hydrochloride (Adriblastina®, Pfizer, Brazil; 1 mg $kg^{-1}.d^{-1}$) during 10 consecutive days, totaling 10 mg kg^{-1}. Then, they were euthanized with an overdose of sodium thiopental (120 mg kg^{-1}, *ip*) one (DOX1), two (DOX2) or four (DOX4) weeks after the last drug injection. Control group received 0.9% saline *ip* injections instead, and rats were euthanized four weeks after the end of saline administration. The number of animals for each experiment is described below.

Isolated heart preparation

Hearts (n = 8 per group) were excised after a mid-line thoracotomy and were immediately cannulated through the aorta according to the method of Langendorff [8] and perfused *via* the coronary circulation at a constant flow rate of 10 $ml.min^{-1}$ with modified Krebs-Henseleit solution (mM: 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 10 glucose and 1.8 $CaCl_2$, pH 7.2; oxygenation with 95% O_2/5% CO_2, 36 ± 0.5 °C). A saline-filled latex balloon connected to a pressure transducer (AVS Projetos, São Paulo, Brazil) was inserted into the left ventricle through the left atrium and adjusted to an end-diastolic pressure of 5–10 mmHg. After 30 min of stabilization, baseline measurements were recorded, and shortly after, hearts were subjected to pharmacological stress with increasing concentrations of norepinephrine (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M *in bolus*, Sigma Aldrich, São Paulo, Brazil). Left ventricle pressures were recorded for the calculation of the following variables (ANCAD software, AVS Projetos, Sao Paulo, Brazil): left ventricle developed pressure, heart rate, and peak positive and negative differentials of pressure change with time (+ d*P*/d*t* and −d*P*/d*t*, respectively).

Blood collection and left ventricle isolation

In another subset of animals (n = 8 per group), the rats were anesthetized with an overdose of sodium thiopental (120 mg kg^{-1}, *ip*). Blood was collected from the abdominal aorta in heparin-containing tubes, and it was immediately centrifuged at 2000 *g* for 10 min. Plasma was collected and immediately stored at −80 °C. Hearts were quickly excised, weighted, and the left ventricle was isolated and also weighted. The right tibia was isolated and its length was measured with a caliper. Both heart and left ventricle mass were corrected for tibia length (in mm). The left ventricle was immediately frozen in liquid nitrogen and stored at −80 °C or processed for light or transmission electron microscopy, as described below. Total protein concentration was assayed using the BCA assay kit (Bioagency, Sao Paulo, Brazil).

Cardiac troponin I plasma levels

The quantitative determination of cardiac troponin I in plasma was performed by an electrochemiluminescence immunoassay (Cobas e 601, Roche Diagnostic).

Oxidative stress in left ventricle homogenate

Lipid peroxidation

Byproducts of lipid peroxidation were measured by thiobarbituric acid reactive substances spectrophotometric assay [9]. Results are expressed as pmol mg^{-1} of protein.

Protein carbonylation

Oxidative damage to protein structures was assessed by determination of carbonyl groups based on the reaction with dinitrophenylhydrazine (Sigma, St. Louis, MO, USA) using the method of Wehr and Levine [10]. Carbonyl contents were determined from the absorbance at 370 nm.

Activity of antioxidant enzymes

The activity of the antioxidant enzymes was assessed by spectrophotometric assays. Superoxide dismutase (SOD) activity was determined by the ability of the enzyme to inhibit the autoxidation of pyrogallol [11]. Catalase activity was estimated by the rate of decomposition of hydrogen peroxide [12]. Glutathione peroxidase (GPx) activity was assayed according to the protocol of Paglia and Valentine [13] based on the rate of NADPH disappearance.

Left ventricle fibrosis

Left ventricle fragments were fixed by immersion in freshly prepared 4% (w/v) formaldehyde in 0.1 M phosphate buffer pH 7.2. After embedding in paraffin, blocks were sectioned and stained with picrosirius red for collagen content.

Transmission electron microscopy

Left ventricle fragments were washed in PBS and fixed for 1 h in a solution containing 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) plus 3.5% sucrose. Then, the samples were washed for 10 min in the same buffer. This wash step was repeated 3 times. The tissue was postfixed for 1 h with a 1% osmium tetroxide solution in 0.1 M sodium cacodylate buffer (pH 7.2) plus 3.5% sucrose, dehydrated in an acetone series (30%, 50%, 70%, 90% and 100%) and embedded in Poly/Bed®812 resin (Ted Pella Inc, Redding, CA, USA). After polymerization, ultrathin sections were obtained and contrasted with uranyl acetate-lead citrate for ultrastructural observation. Sections were viewed in a Zeiss EM906 microscope (Zeiss, Jena, Germany).

Apoptosis detection

The extent of apoptosis was assessed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Click-iT® Plus TUNEL Assay, Life Thermo Fisher Scientific Inc, USA) in formalin-fixed paraffin-embedded left ventricle sections. The

number of TUNEL-positive cardiomyocyte nuclei in a frame of known area was counted in 5 fields per animal. The test system was established using the Image Pro Plus 5 (Media Cybernetics, Inc).

Statistical analysis

Data are presented as mean ± SEM. Differences between groups were assessed by ANOVA with Holm-Sidak's multiple comparisons test, after testing its assumptions. Langendorff derived data were compared by a 2-way repeated measure ANOVA (group x norepinephrine concentration). The significance level was set at 5%. Statistical analysis was performed using the software GraphPad Prism 6 (GraphPad Inc., CA, USA).

Results

General data

There was a 30% mortality in each of the three DOX groups. Body mass, left ventricular (LV) and heart mass are shown in Table 1. The results demonstrate that doxorubicin significantly reduces body mass, heart and left ventricle mass one week after the end of doxorubicin administration (DOX1), that was maintained thereafter (DOX2 and DOX4). Left ventricle and heart mass were corrected by right tibia length (mm).

Left ventricle function

As shown in Fig. 1, basal left ventricle contractility (assessed by LVDP and + dP/dt) and relaxation (assessed by -dP/dt) were significantly impaired only after four weeks following the end of doxorubicin administration (DOX4).

On the other side, a reduced response to adrenergic stimuli developed earlier. There was an impairment in both left ventricle contractility and relaxation in response to norepinephrine two weeks after the end of DOX administration, that was further deteriorated at week four (Fig. 1B, D, and C).

Oxidative stress

Left ventricle lipid peroxidation was increased shortly after the end of doxorubicin administration in comparison with control group, and remained elevated up to week four (Table 2). On the other hand, protein oxidative damage was evident only after four weeks (DOX4). The activity of the antioxidant enzymes SOD and CAT did not change following doxorubicin administration. In contrast, glutathione peroxidase activity was significantly higher in DOX4 compared with all other groups.

Left ventricle ultrastructural analysis by transmission electron microscopy

Doxorubicin administration resulted in marked myocardial damage, as shown in Fig. 2. Ultrastructural changes are already seen one week after the last doxorubicin injection. In DOX1 (Fig. 2B) and DOX2 (Fig. 2C), it is possible to observe a thickening of Z line, formation of intermyofibrillar and intermitochondrial vacuoles and mitochondrial derangement, in comparison with normally arranged cardiomyocytes and intact mitochondria in control group (Fig. 2A). A more extensive damage is observed in DOX4 group (Fig. 2D). After four weeks of doxorubicin treatment, mitochondria are shown edematous, the nuclei have apoptotic aspect with chromatin disorganization, and a large area of intracellular vacuoles is evidenced.

Plasma levels of cardiac troponin I

Increased cardiac troponin I plasma levels followed ultrastructural damage observed in the left ventricle. Cardiac troponin I levels were significantly increased at one and two weeks after the end of doxorubicin injection compared to the control group, and further increased after four weeks (Fig. 3).

Left ventricle fibrosis and apoptosis

Left ventricle interstitial fibrosis was progressively increased after the end of doxorubicin administration, as shown by increased picrosirius red staining (Fig. 4). In cardiomyocytes of control group, TUNEL staining was absent. The number of TUNEL-positive nuclei was gradually increased along the time, but a statistically significant difference was observed only in DOX4 compared with control group (Fig. 5).

Discussion

Here, we aimed to characterize the time course of cardiac structural and functional changes induced by doxorubicin. One important finding of the study was that doxorubicin causes early morphological damage, systemically manifested as increased plasma levels of cardiac troponin I, which occurs before any evidence of cardiac dysfunction. The relevance of this finding is that cardiac assessment of patients after doxorubicin chemotherapy should not be performed only after the development of symptoms, since cardiac dysfunction at rest is only observed when massive cardiac structural damage has already taken place.

The relative new findings that doxorubicin-induced cardiac damage can be reversed when early intervened [7] renewed the interest in better understanding the progression of the deleterious cardiac effects of this drug. Our results confirmed that the detection of cardiac troponins in the bloodstream may be useful as a highly specific marker of cardiac damage. The changes in systemic cardiac troponins mirrored the magnitude of damage in the ultrastructure of the left ventricle. In addition, this reinforces the recommendation of the European guidelines of measuring cardiac biomarkers at baseline and during follow-up to detect cardiotoxicity [1]. It is important to mention that there is a lack of sufficient evidence to establish the significance of subtle rises in cardiac biomarkers [1]. We realize that it is important to weight the limitation of translating basic to clinical findings, but our results suggest that it may be necessary to intervene soon after any increase in cardiac biomarkers, as it may precede a massive deterioration in cardiac structure and function.

Doxorubicin cardiotoxicity has been traditionally classified into two forms: early or late onset [1]. Currently, it is being debated

Table 1
Body mass (BM), heart and left ventricle (LV) mass of the experimental groups.

	Control	DOX1	DOX2	DOX4
BM (g)	480 ± 3	431 ± 3*	393 ± 2*	374 ± 2*
Heart (g·mm^{-1})	0.042 ± 0.001	0.039 ± 0.003*	0.036 ± 0.003*	0.037 ± 0.001*
LV (g·mm^{-1})	0.020 ± 0.001	0.018 ± 0.001*	0.019 ± 0.001*	0.019 ± 0.001*

* Different from control group ($p \leq 0.05$).

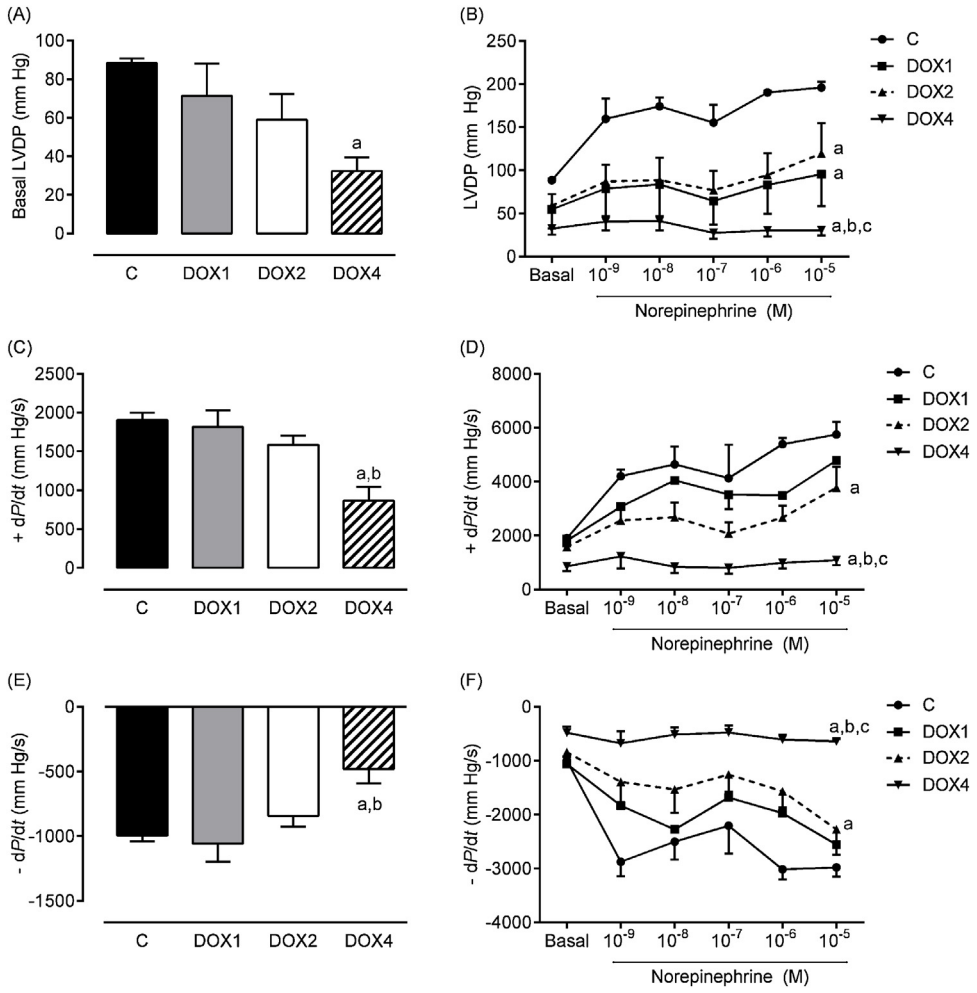

Fig. 1. Effects of doxorubicin on basal cardiac hemodynamics (left - A,C,E) and its response to norepinephrine (right - B,D,F). LVDP, left ventricle developed pressure. [a] Different from control group, [b] Different from DOX1 group, [c] Different from DOX2 group ($p \leq 0.05$)

Table 2
Biomarkers of oxidative stress.

	Control	DOX1	DOX2	DOX4
SOD ($U\,mL^{-1}$)	0.101 ± 0.017	0.108 ± 0.044	0.104 ± 0.058	0.121 ± 0.030
GPx ($mmol\,min^{-1}\,mg^{-1}$)	0.081 ± 0.015	0.076 ± 0.018	0.086 ± 0.018	0.128 ± 0.039 [a,b,*]
CAT ($U\,mg^{-1}$)	3.5 ± 2.1	5.0 ± 2.7	5.6 ± 2.2	4.5 ± 2.4
TBARS ($pmol\,MDA\,mg^{-1}$)	132.5 ± 6.5	229.2 ± 18.2 [a]	214.1 ± 16.5 [a]	231.7 ± 15.3 [a]
Carbonyl groups ($nmol\,mg^{-1}$)	0.322 ± 0.07	0.341 ± 0.09	0.366 ± 0.08	0.802 ± 0.17 [a]

CAT, catalase; GPx, glutathione peroxidase; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.
[a] Different from control group.
[b] Different from DOX1 group.
[*] Different from DOX2 group ($p \leq 0.05$).

whether this classification reflects two different entities or the progression of a single process diagnosed at different times. Ours and others findings suggest that the second hypothesis is more plausible. Firstly, doxorubicin causes a subclinical myocardial damage that then progresses to a decline in left ventricle ejection fraction and, eventually, heart failure [7]. Indeed, we have observed an intense cardiomyocyte disarray soon after the end of doxorubicin administration, but cardiac contractile dysfunction was evident only after a longer period. Ultrastructural changes included a thickening of Z line, formation of intermyofibrillar and intermitochondrial vacuoles and mitochondrial derangement, and these changes were aggravated with time. These massive structural changes are characteristic of doxorubicin administration [14] and, as stated before, despite extensive research, the mechanisms are still debated. Increased oxidative stress is likely to play a major role, as it has been shown to activate proteolytic, autophagic [15] and both extrinsic and intrinsic apoptotic [16] pathways. Our findings are in agreement with this statement, as we observed increased lipid oxidative damage shortly after doxorubicin treatment, probably due to increased ROS generation without any compensatory increase in the activity of antioxidant enzymes. Understanding the sequence of events relating reactive oxygen/nitrogen species production, the regulation of expression and activity of antioxidant enzymes, and resultant oxidative stress/damage is challenging. In addition, the responses may be quite different depending on cell type, reactive oxygen species produced,

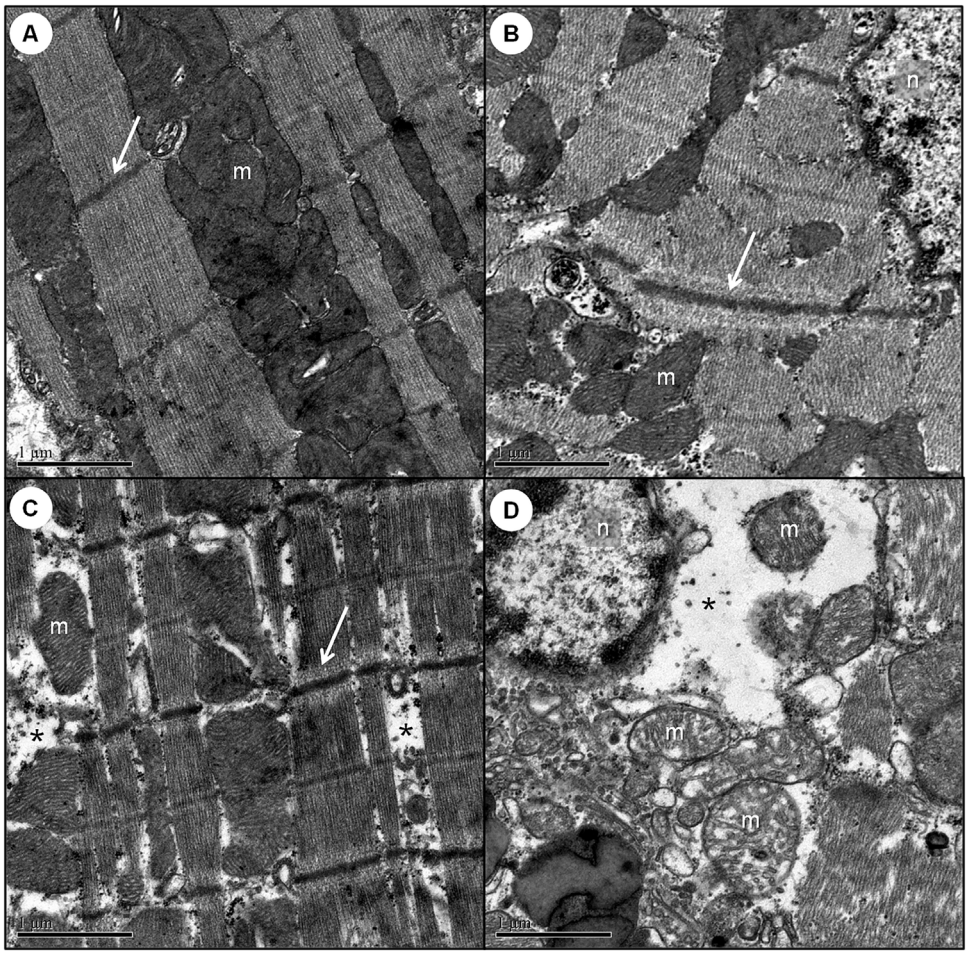

Fig. 2. Transmission electron micrography of left ventricle myocytes from control (A), DOX1 (B), DOX2 (C) and DOX4 (D) groups. Magnification x20,000. White arrows, Z line; * intracellular vacuoles; m, mitochondria; n, nucleus.

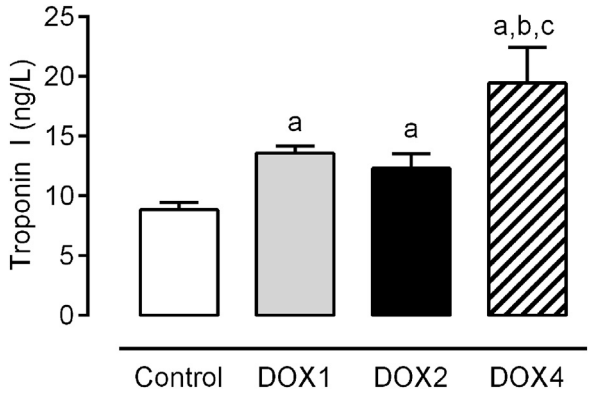

Fig. 3. Cardiac troponin I levels measured in plasma. [a] Different from control group, [b] Different from DOX1 group, [c] Different from DOX2 group ($p \leq 0.05$).

and timing after insult. Based on hormesis principle, an increase in oxidative stress would theoretically lead to an increase in antioxidant defense. However, we found increased lipid oxidative damage at week 1, and the increase in glutathione peroxidase activity was observed only at week 4 after doxorubicin exposure. Similar findings were found by Rabelo et al. [17] that demonstrated increased levels of lipid peroxidation and a reduction in glutathione peroxidase activity in heart tissue from rats three weeks after a cumulative doxorubicin dose of 13 mg/kg. On the other side, acute or short-term studies usually show an increase in gene or mRNA expression of antioxidant enzymes, but an absence of change in their activities. For instance, it has been shown that 24 h of doxorubicin exposure led to an increase in the expression of the genes GSTM1 and GSTA-1, which encodes glutathione-related enzymes, but did not change glutathione peroxidase activity in HeLa cells [18].

It was also noted an early reduction in both heart and left ventricle mass. This finding is in accordance with previous studies that have also shown a significant decrease in heart mass [19], thinning of left ventricular septal wall and cardiomyocyte fiber diameter [19], suggesting that doxorubicin induces cardiac atrophy. In humans, noninvasive estimates of left ventricle mass obtained from cardiac magnetic resonance imaging have shown that left ventricle mass index is inversely related to doxorubicin dose in patients after 88 months of chemotherapy on average [20]. In addition, a smaller left ventricle mass index was associated with a greater incidence of cardiac adverse events in this same cohort that was followed for 27 months on average [20]. Therefore, the measurement of both heart and left ventricle mass be included in the routine assessment of cardiac health following doxorubicin chemotherapy. Our results also suggest that early cardiac atrophy induced by doxorubicin is likely to be driven by causes other than apoptosis, as increased apoptotic nuclei were only observed at four weeks after the end of doxorubicin administration. Similar findings have been reported by Zhu et al. [19], who demonstrated that soon after starting doxorubicin treatment in juvenile mice a reduction in heart mass was evident, but increased levels of collagen deposition

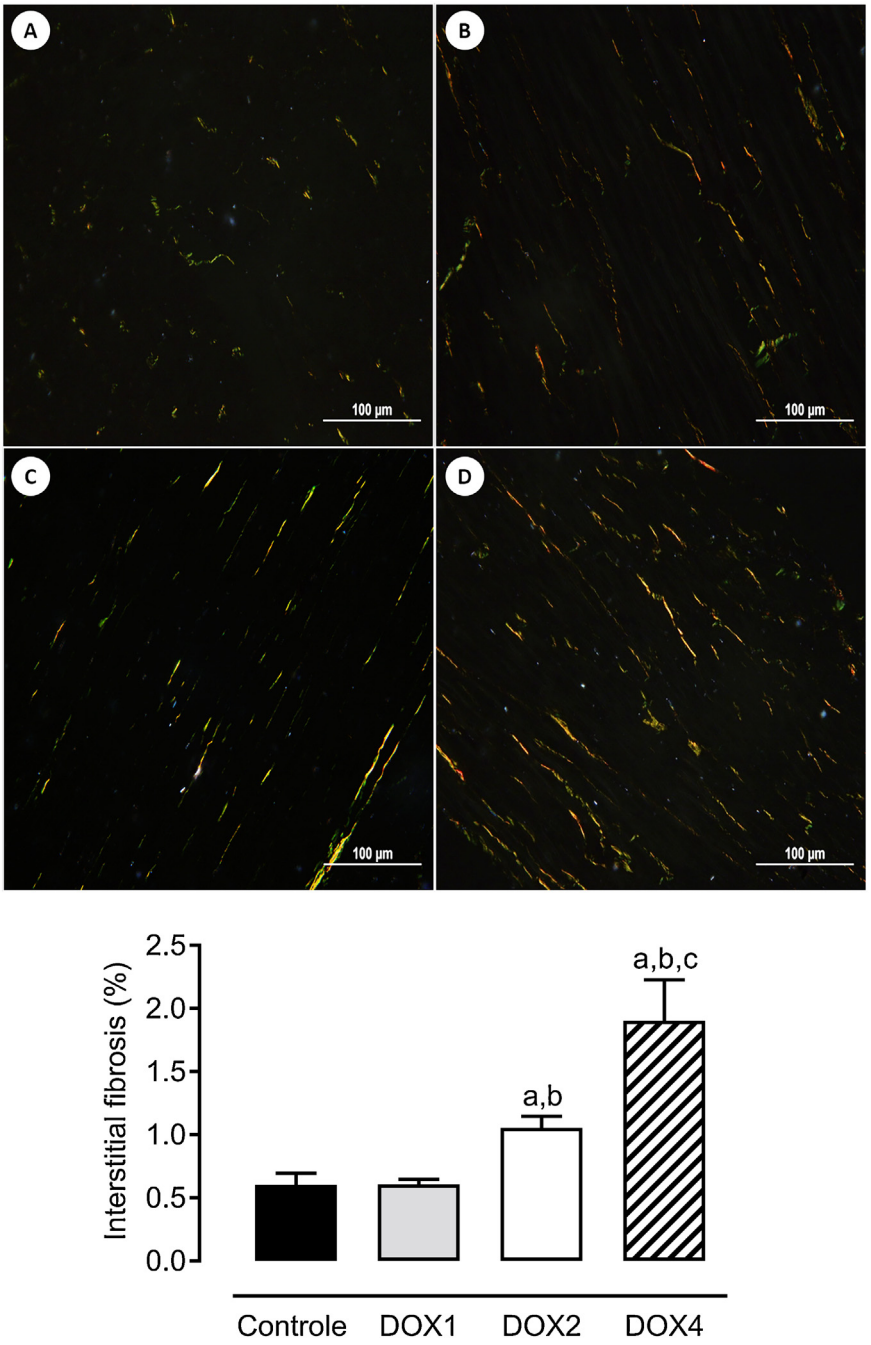

Fig. 4. Top, representative light photomicrographs of picrosirius red stained sections of the left ventricle from control (A), DOX1 (B), DOX2 (C) and DOX4 (D) groups. Bottom, interstitial fibrosis quantitation. The magnification was 40×. [a] Different from control group, [b] Different from DOX1 group, [c] Different from DOX2 group ($p \leq 0.05$) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

and cardiomyocyte apoptosis were shown after 13 weeks. Decreased heart mass may be caused by an imbalance in protein turnover, with increased proteolysis *via* activation of ubiquitin-proteasome [21] and calpain system [22], as well as increased autophagy [15].

This initial cell damage was followed by an adverse cardiac remodeling, demonstrated by increased accumulation of extracellular matrix and collagen deposition. Doxorubicin-induced cardiac fibrosis may be due to increased proteolysis and apoptotic cell damage [23], as well as increased oxidative stress. Excessive ROS generation has been demonstrated to upregulate TGF-β/SMAD3 pro-fibrotic pathway in rats treated with doxorubicin [24].

As a consequence of these massive structural deleterious changes, left ventricle systolic and diastolic dysfunction ensues, but impairment in basal state was only noted after four weeks. Similar findings were recently reported by Rodrigues et al. [25] in a rabbit model of cardiomyopathy induced by doxorubicin. They demonstrated that cardiac dilation and important molecular abnormalities, including increased myocardial oxidative stress, fibrosis, apoptosis, a shift in the stiff N2B titin isoform to the more compliant N2BA and increased aggregation of histidine-rich glycoprotein fragment, were evident one week after the last drug infusion despite preserved systolic function assessed by echocardiography.

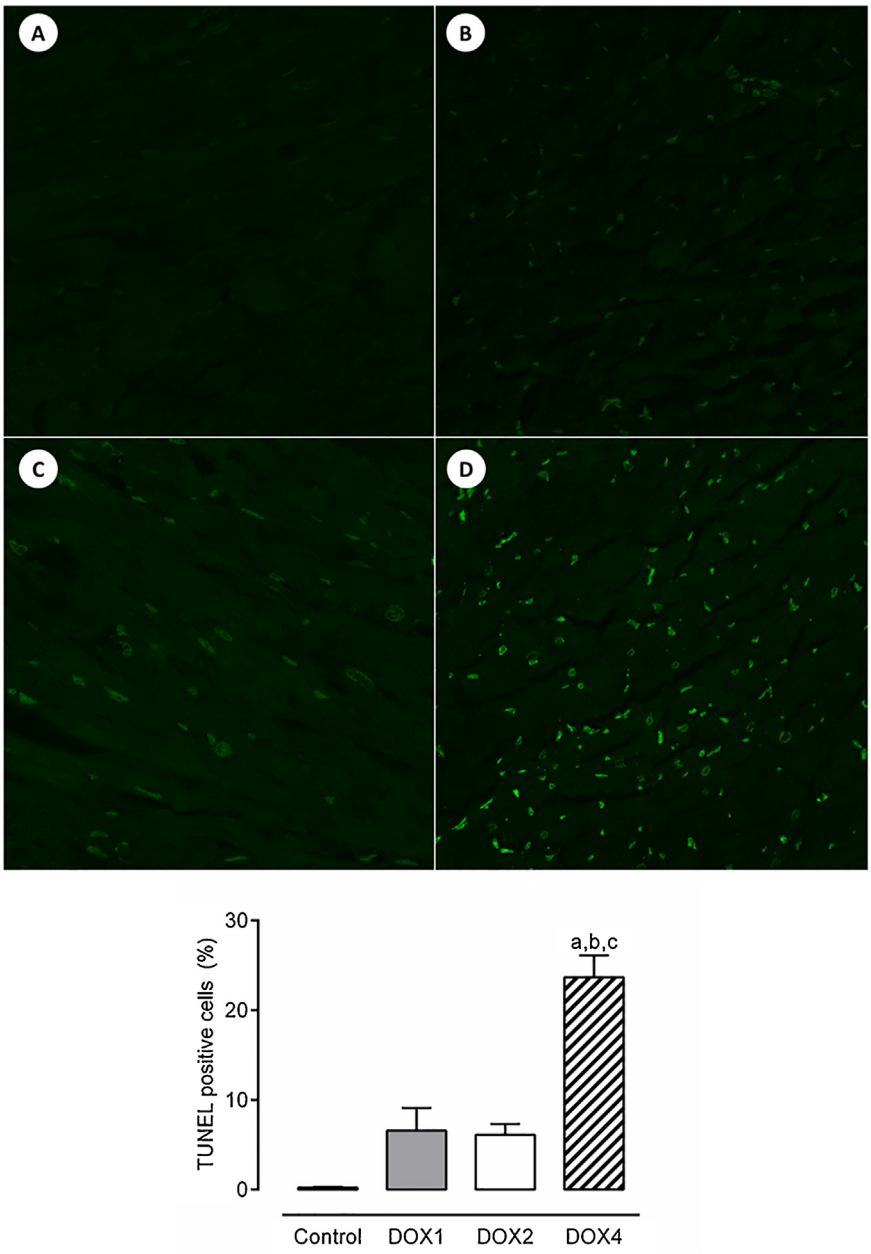

Fig. 5. Top, representative fluorescent micrographs of TUNEL stained sections of left ventricle from control (A), DOX1 (B), DOX2 (C) and DOX4 (D) groups. Bottom, quantitation of TUNEL positive cells. The magnification was 40 ×. [a] Different from control group, [b] Different from DOX1 group, [c] Different from DOX2 group ($p \leq 0.05$).

Left ventricular dysfunction in the absence of pharmacological challenge was noticed after four weeks, but, importantly, we observed a reduction in the response to the lowest concentration of norepinephrine tested in all animals from doxorubicin groups. In comparison with control group, animals from both DOX1 and DOX2 groups displayed a 40% lower left ventricle developed pressure, + dP/dt and - dP/dt with norepinephrine infusion. Studies have demonstrated that altered sympathetic regulation seems to be a characteristic feature of doxorubicin-induced cardiomyopathy [26–28]. In end-stage congestive heart failure caused by doxorubicin treatment, inotropic support with isoproterenol failed to revert cardiac decompensation [29]. Adrenergic refractoriness was also demonstrated in rodent models [26,27], but none of them have demonstrated that this hyporesponsiveness is of so early onset. Impairment in beta-adrenergic signaling seems to occur at post-synaptic level, since doxorubicin does not seem to affect norepinephrine release from pre-synaptic sympathetic neurons [26,27]. Doxorubicin effects on myocardial β-adrenergic receptor density are not consistent, with some demonstrating a decrease in β_1 [30,31], others no change in any β-adrenergic receptor [32], and others an increase in β_2 subtype [28]. But there is an agreement that adenylate cyclase activity and cyclic AMP generation are both impaired after doxorubicin treatment [32,33]. These findings may allow us to suggest that dobutamine stress test could be used in the assessment of cardiac function in patients under doxorubicin chemotherapy, since impairment in catecholamine-induced response occurs earlier than resting cardiac dysfunction takes place.

Our results corroborate the current view that doxorubicin cardiotoxicity occurs in a “continuum”. Here, it began with structural damage probably caused by excessive reactive species production and an early cardiac adrenergic refractoriness that progressed to activation of apoptotic pathways and cardiac

remodeling that ended with a severe impairment of both contractile and relaxation properties. As the consolidated view of an irreversible cardiac injury caused by doxorubicin is being challenged [7], understanding the progression of morphological and functional changes caused by doxorubicin may allow a proper timing of initiation of prophylactic treatment.

Conflict of interest

None.

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