Acute Doxorubicin-Induced Cardiotoxicity is Associated with Matrix Metalloproteinase-2 Alterations in Rats

Bertha Furlan Polegato\textsuperscript{a} Marcos Ferreira Minicucci\textsuperscript{a} Paula Schmidt Azevedo\textsuperscript{a} Robson Francisco Carvalho\textsuperscript{b} Fernanda Chiuso-Minicucci\textsuperscript{c} Elenize Jamas Pereira\textsuperscript{a} Sergio Alberto Rupp Paiva\textsuperscript{a} Leonardo Antônio Mamede Zornoff\textsuperscript{a} Marina Politi Okoshi\textsuperscript{a} Beatriz Bojikian Matsubara\textsuperscript{a} Luiz Shiguero Matsubara\textsuperscript{a}

\textsuperscript{a}Internal Medicine Department, Botucatu Medical School, Univ Estadual Paulista (UNESP), Brazil
\textsuperscript{b}Morphology Department, Botucatu Biosciences Institute, Univ Estadual Paulista (UNESP), Brazil
\textsuperscript{c}Microbiology and Immunology Department, Botucatu Biosciences Institute, Univ Estadual Paulista (UNESP), Brazil

Key Words
Isolated heart study • Cytokines • Phospholamban • Ryanodine receptor • Serca-2a • Matrix metalloproteinase • Doxorubicin • Acute cardiotoxicity

Abstract
Background: Doxorubicin can cause cardiotoxicity. Matrix metalloproteinases (MMP) are responsible for degrading extracellular matrix components which play a role in ventricular dilation. Increased MMP activity occurs after chronic doxorubicin treatment. In this study we evaluated \textit{in vivo} and \textit{in vitro} cardiac function in rats with acute doxorubicin treatment, and examined myocardial MMP and inflammatory activation, and gene expression of proteins involved in myocyte calcium transients. Methods: Wistar rats were injected with doxorubicin (Doxo, 20 mg/kg) or saline (Control). Echocardiogram was performed 48 h after treatment. Myocardial function was assessed \textit{in vitro} in Langendorff preparation. Results: In left ventricle, doxorubicin impaired fractional shortening (Control 0.59±0.07; Doxo 0.51±0.05; p<0.001), and increased isovolumetric relaxation time (Control 20.3±4.3; Doxo 24.7±4.2 ms; p=0.007) and myocardial passive stiffness. MMP-2 activity, evaluated by zymography, was increased in Doxo (Control 141338 ± 8924; Doxo 188874 ± 7652 arbitrary units; p<0.001). There were no changes in TNF-\textgreek{a}, INF-\textgreek{\gamma}, IL-10, and ICAM-1 myocardial levels. Expression of phospholamban, Serca-2a, and ryanodine receptor did not differ between groups. Conclusion: Acute doxorubicin administration induces \textit{in vivo} left ventricular dysfunction and \textit{in vitro} increased myocardial passive stiffness in rats. Cardiac dysfunction is related to myocardial MMP-2 activation. Increased inflammatory stimulation or changed expression of the proteins involved in intracellular calcium transients is not involved in acute cardiac dysfunction.
Introduction

Doxorubicin is an antineoplastic drug used in the treatment of solid tumors and hematologic malignancies [1]. Despite its wide use, doxorubicin has severe side effects with cardiotoxicity being the most important [2-4]. Cardiotoxicity is classified into four clinical stages, acute, subacute, chronic, and late, according to elapsed time between drug administration and toxicity symptom onset [3, 4]. Chronic toxicity has received more attention and has been widely studied; it has been associated with dilated cardiomyopathy and typical features of heart failure in 4-23 % of treated patients [2, 3]. Most patients remain asymptomatic during and after drug infusion, however electrocardiographic abnormalities such as QT interval prolongation and nonspecific ventricular repolarization changes can acutely appear in approximately 11 % of patients [5, 6]. Despite the acute clinical form being asymptomatic in most cases, myocardial injury may begin immediately after drug infusion [7-10].

Matrix metalloproteinases (MMP) are zinc-dependent proteolytic enzymes responsible for extracellular matrix component degradation. They play a fundamental role in cardiac remodeling and ventricular dilation in several myocardial injury models [11]. Different stimuli such as inflammatory and neurohormonal activation, transient intracellular calcium changes, increased oxidative stress, and myocardial stretching can exacerbate MMP activation [11]. Increased MMP gene expression and activity have been seen after chronic doxorubicin treatment [12]. However, acute MMP activation after doxorubicin has been poorly addressed [13]. In this study we demonstrated that MMP-2 is acutely activated after doxorubicin treatment and showed that MMP-2 activation is combined with in vivo left ventricular systolic dysfunction and increased in vitro passive myocardial stiffness in rats. We also demonstrated that acute left ventricular dysfunction is not associated with myocardial inflammatory activation or changed gene expression of proteins involved in myocyte intracellular calcium transients.

Material and Methods

All experiments and procedures were approved by the Ethics Committee of Botucatu Medical School, UNESP, Brazil (Protocol number 666/08). All rats were housed in a room at a controlled temperature of 23°C and kept on a 12-hour light/dark cycle. Food and water were supplied ad libitum.

Thirty-week-old male Wistar rats were treated with a single dose of doxorubicin (Doxo group, 20 mg/kg, intraperitoneal injection, n=18) or an equivalent sterile saline volume (Control group, n=17). All rats were subjected to echocardiography before and 48 h after treatment. After final echocardiogram, rats were anesthetized and hearts removed for isolated heart studies (Control group, n=12; Doxo group, n=13). The hearts subjected to isolated heart study were not used in other analyses as in vitro perfusion can change myocardial tissue properties.

Echocardiographic study

Animals were lightly anesthetized with an intraperitoneal injection of ketamine (50 mg/kg) and xylazine (1 mg/kg). Echocardiography was performed by the same blinded examiner using a Philips HDI 5000 apparatus with a 7-12 MHz multifrequency transducer. All parameters were obtained from parasternal long and short axis and apical four chambers [14-17]. Structural variables analyzed by Doppler-echocardiography were: left atrial diameter, left ventricular (LV) diastolic posterior wall thickness, and LV diastolic and systolic diameters. Diastolic function was evaluated by transmitral Doppler E wave, A wave, E/A ratio, and isovolumetric relaxation time. Systolic function was analyzed by ejection fraction, LV fractional shortening, and cardiac output. Heart rate was calculated from the distance between two consecutive cardiac cycles; all values were obtained from the average of three consecutive beats.
Isolated heart study

After echocardiographic study, rats were anesthetized with sodium thiopental (50 mg/kg, i.p.) and given heparin (2000 IU, i.p.). The chest was opened by median sternotomy under artificial ventilation. The ascending aorta was isolated and cannulated for retrograde perfusion. The heart was quickly removed and transferred to a perfusion apparatus (model 830 Hugo Sachs Eletronick-Green-Strasse). Retrograde perfusion was established with filtered oxygenated Krebs-Henseleit solution (composition, in mmol/L: NaCl 118.5; KCl 4.69; CaCl$_2$ 2.52; MgSO$_4$ 1.16; KH$_2$PO$_4$ 1.18; glucose 5.50; NaHCO$_3$ 25.88; and mannitol 8) [18] maintained at constant temperature (37°C) and perfusion pressure (75 mmHg). All hearts were paced at 200 to 250 beats/min. Latex balloon was inserted in left ventricle and volume inside the balloon was increased progressively. Pressure values, maximum LV pressure decrease rate and maximum LV pressure development rate were recorded. Procedures and measurements were performed according to a previously described method [19-21]. To assess myocardial diastolic stiffness in hearts with different left ventricular weight and size, stress (g/cm$^2$) and strain (%) at the LV midwall were calculated assuming the LV to be a thick-walled sphere. The equations were as follows:

\[
\text{stress} = \left[1.36xLVPxLV^{2/3}\right]/\left[\left(LVV+0.943xLVW\right)^{2/3}-LVV^{2/3}\right];
\]

\[
\text{strain} = \left[LVV^{1/3}+(LVW+0.943xLVW)^{1/3}\right]/\left[V0^{1/3}+(V0+0.943xLVW)^{1/3}\right]-1\times100
\]

where $x$ is the multiplication sign, $LV$ is LV volume (mL), $V0$ is LVV at an end-diastolic pressure of 0 mm Hg, $LVW$ is LV weight (g), and $LVP$ is LV end-diastolic pressure (mm Hg).

Diastolic function was analyzed by maximum LV pressure decrease rate (-dP/dt), percentage of variation in LV volume required to increase diastolic pressure from 0 to 20 mmHg ($\Delta V_{20}$, index of LV compliance), and percentage of myocardial strain caused by a diastolic stress of 20 g/cm$^2$ (myocardial elasticity index). Systolic function was analyzed by maximum LV pressure development rate (+dP/dt), and maximum developed pressure [21].

Morphometric analysis

LV transverse sections were fixed in 10 % buffered formalin and embedded in paraffin. Five micron thick sections were stained with collagen-specific picrosirius red dye (Sirius red F3BA in aqueous saturated picric acid) [22]. Interstitial collagen volume fraction was determined for the entire cardiac section by analyzing digital images under polarized light. Myocardial tissue components were identified according to the following staining patterns: red for collagen fibers and black for myocytes and interstitium. The collagen volume fraction was calculated as the sum of all connective tissue areas divided by the sum of all connective tissue and myocyte areas. Perivascular collagen was excluded from analysis [15]. On average, 35 microscopic fields were evaluated in each heart. Measurements were performed using a Leica microscope attached to a video camera connected to a computer equipped with image analysis software (Image-Pro Plus 3.0, Media Cybernetics, Silver Spring, MD, USA).

ELISA

Cardiac tissue samples (60 mg) were homogenized and solubilized in 50 mM potassium phosphate buffer pH 7.4, 0.3 M sucrose, 0.5 mM DTT, 1 mM EDTA pH 8.0, 0.3 mM PMSF, 10 mM NaF, and 1:100 protease inhibitor. Levels of interferon (IFN)-$\gamma$, tumor necrosis factor (TNF)-$\alpha$, interleukin (IL)-10, and intercellular adhesion molecule (ICAM)-1 were evaluated according to manufacturer instructions (R & D Systems, Minneapolis, MN, USA).

Real-time quantitative reverse transcription–polymerase chain reaction (RT-qPCR)

Ribonucleic acid (RNA) was extracted from LV using TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA was quantified with a spectrophotometer (NanoDrop ™ 2000 Spectrophotometer Thermo Scientific, Nanodrop Technologies). Absorbance was analyzed for wavelengths of 230, 260, and 280 nm. All samples had 260/230 nm and 260/280 nm absorbance ratios higher than 1.8. RNA integrity was verified by electrophoresis on 1 % agarose gel [23]. RNA was reverse transcribed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA, USA) as recommended by the manufacturer. Aliquots of cDNA were then submitted to PCR reaction in the StepOne Plus platform (Applied Biosystems, CA, USA) using TaqMan MGB-probe (FAM TaqMan Universal PCR Master Mix, Applied Biosystems, CA, USA), and primers (TaqMan Gene Expression Assay GEx, Applied Biosystems, CA, USA) specific to each gene: phospholamban (Rn01434045_m1), sarcoplasmic reticulum calcium ATPase (Serca-2a; Rn00568762_m1),...
ryanodine receptor (Rn01470303_m1), and cyclophilin (Rn00690933_m1). The samples were amplified in triplicate and results normalized to reference gene cyclophilin. Expression levels were calculated by the 2^ΔΔCT method.

**Zymography**

MMP-2 was analyzed as per Tyagi et al [24, 25]. In brief, samples were diluted in extraction buffer with 50 mM Tris pH 7.4, 0.2 M NaCl, 0.1 % Triton X, and 10 mM CaCl₂. Protein in samples was quantified by Bradford method. Samples with 20 µg of protein were then diluted in application buffer with 0.5 M Tris pH 6.8, 50 % glycerol, and 0.05 % bromophenol blue, and loaded into wells of 8 % SDS-polyacrylamide containing 1 % gelatin. Electrophoresis was run in a Bio-Rad apparatus at 80 V for 2 h. Gel was removed, washed with 2.5 % Triton-X-100, and washed with 50 mM Tris pH 8.4. Gel was then incubated at 37 °C overnight in activation solution with 50 mM Tris pH 8.4, 5 mM CaCl₂ and ZnCl₂. Staining was performed for 2 h with 0.5 % comassie blue and destaining in 30 % methanol and 10 % acetic acid at room temperature on a rotatory shaker. To identify the gel activity of MMP-2, we used recombinant mouse/rat MMP-2 standard (R&D Systems) as a positive control. The gels were photographed by Gel Logic 6000 Pro (Carestream Health Inc.) and the intensity of gelatinolytic action (clear bands) was analyzed by GelPro 3.1.

**Statistical analysis**

Data are expressed as mean and standard deviation or median and 25th and 75th percentiles. Comparisons between the groups were performed by Student’s t test, for values with normal distribution, or Mann-Whitney test, for values with non-normal distribution. Body weight before and after treatment was analyzed by paired Student’s t test. Analysis of covariance (ANCOVA) was performed to evaluate systolic functional variables without the influence of heart rate and body weight [26]. Significance level was set at 5 %.

**Results**

Body weight did not differ between groups before treatment (Control 329±14; Doxo 333±16 g; p>0.05). After treatment, body weight decreased in Doxo (initial 333±16; final 318±19 g; n=15; p<0.001) and increased in Control group (initial 329 ±14; final 338±21 g; n=17; p=0.019). LV ventricle weight did not differ between groups [Control 0.78±0.06 (n=12); Doxo 0.75±0.06 g (n=13); p=0.20].

Before treatment, all echocardiographic variables were similar between groups (data not shown). Echocardiographic data after treatment are shown in Table 1. LV diastolic and systolic diameters, LV diastolic posterior wall thickness, E wave, A wave, and E/A ratio did not differ between groups. Isovolumetric relaxation time in absolute and normalized to heart rate values was increased in the Doxo group. LV fractional shortening, LV ejection fraction, and cardiac output were lower in Doxo. After adjustment for heart rate

| Table 1. Echocardiographic data after treatment. HR: heart rate; LA: left atrium diameter; LVDD and LVSD: left ventricular (LV) diastolic and systolic diameters, respectively; LVPWTR: LV diastolic posterior wall thickness; IVRT: LV isovolumetric relaxation time; LVFS: LV fractional shortening; LVEF: LV ejection fraction, CO: cardiac output. Values are mean ± standard deviation or median and 25th and 75th percentile; p value: result of Student’s t* or Mann-Whitney test# |
|-----------------|-----------------|-----------------|
|                  | Control (n=17)  | Doxo (n=15)     |
| **HR (bpm)**    | 346 ± 65        | 301 ± 46        | 0.03* |
| **LA (mm)**     | 4.22 ± 0.5      | 4.01 ± 0.58     | 0.29* |
| **LVDD (mm)**   | 7.2 ± 0.52      | 6.82 ± 0.64     | 0.08* |
| **LVSD (mm)**   | 2.97 ± 0.66     | 3.36 ± 0.62     | 0.10* |
| **LVPWTR (mm)** | 1.31 ± 0.15     | 1.33 ± 0.16     | 0.71* |
| **E wave (cm/s)** | 77.9 ± 15       | 68.7 ± 10.5     | 0.06* |
| **A wave (cm/s)** | 63.2 ± 19.7    | 51.9 ± 14.3     | 0.08* |
| **E/A**         | 1.19 (1.1-1.47) | 1.25 (1.17-1.53) | 0.29# |
| **IVRT (ms)**   | 20.3 ± 4.3      | 24.7 ± 4.2      | 0.007* |
| **IVRT/HR**     | 44.8 ± 4.9      | 49.5 ± 4.3      | 0.007* |
| **LVFS**        | 0.59 ± 0.07     | 0.51 ± 0.05     | <0.001* |
| **LVEF**        | 0.93 ± 0.03     | 0.88 ± 0.04     | <0.001* |
| **CO (ml/min)** | 72.7 ± 11.7     | 55.3 ± 16.4     | 0.002* |
and body weight, LV fractional shortening, LV ejection fraction, and cardiac output remained lower in the Doxo group (Table 2).

In the isolated heart study, strain under 20 g/cm² diastolic stress (point marked with * in figure) was significantly lower in the doxorubicin group (p<0.05), characterizing increased passive myocardial stiffness.

![Graph showing stress-strain relationship](image)

**Table 2.** Echocardiographic variables adjusted by body weight and heart rate with covariance analysis (ANCOVA). LVFS: left ventricular fractional shortening; LVEF: LV ejection fraction; CO: cardiac output. Values are mean ± standard error; p value: result of ANCOVA test.

| Variable          | Control (n=17) | Doxo (n=15) | p value |
|-------------------|---------------|-------------|---------|
| LVFS              | 0.59 ± 0.02   | 0.52 ± 0.02 | 0.011   |
| LVEF              | 0.93 ± 0.01   | 0.88 ± 0.01 | 0.003   |
| CO (ml/min)       | 70.9 ± 3.49   | 57.4 ± 3.79 | 0.028   |

**Fig. 1.** Stress-Strain Relationship. Isolated heart stress-strain relationship for control (n=7) and doxorubicin (n=7) groups. Strain under 20 g/cm² diastolic stress (point marked with * in figure) was significantly lower in the doxorubicin group (p<0.05), characterizing increased passive myocardial stiffness.

**Fig. 2.** Myocardial matrix metalloproteinase (MMP)-2 activity. Illustrative electrophoresis gel zymography in myocardial tissue. Columns 1, 3, 5, and 7: Control group; columns 2, 4, 6, and 8: Doxorubicin group; column 9: recombinant MMP-2 standard; column 10: molecular weight standard. White bands correspond to gelatin degradation by MMP-2. MMP-2 activity was identified through the positive control recombinant mouse/rat MMP-2 standard (column 9). Figure shows an increase in MMP-2 activity in the Doxorubicin treated rats.
In morphometric analysis, general myocardial histology was preserved in both Control and Doxo groups. Interstitial collagen volume fraction did not differ between groups [Control 3.43±1.16 (n=17); Doxo 3.82±1.63 % (n=15); p=0.26].

In zymography, the MMP-2 activity was identified by using the positive control recombinant MMP-2 standard, which presented three bands (Fig. 2). The MMP-2 activity was increased in the Doxo group (Table 3). Gene expression of phospholamban, Serca-2a, and ryanodine receptor (Table 4), and myocardial concentration of TNF-α, INF-γ, IL-10, and ICAM-1 (Table 5) did not differ between groups.

### Discussion

In this study we confirmed that MMP-2 is acutely increased after doxorubicin treatment in rats and showed for the first time that MMP-2 activation is combined with left ventricular systolic dysfunction and increased passive myocardial stiffness in rats. We also showed that left ventricular dysfunction is not associated with changes in TNF-α, INF-γ, IL-10, and ICAM-1 or gene expression of the proteins involved in myocyte intracellular calcium transient.

Body weight decreased after doxorubicin treatment (approximately 4.5 %), which agrees with previous studies in rats and mice [27-29]. Body weight loss was probably related to dehydration and hypophagia [30].

Echocardiography showed that relaxation was slower in Doxo than Control, characterized by the increased isovolumetric relaxation time in absolute or heart rate normalized values [14]. Systolic function was depressed as shown by reduced LV fractional shortening, ejection fraction, and cardiac output. Body weight and heart rate can modulate LV diastolic and systolic diameters and systolic functional parameters. We therefore performed covariance analysis to adjust LVFS, LVEF, and CO results for body weight and heart rate. After adjustment, systolic function remained impaired in the Doxo group. These results are consistent with those from studies evaluating acute doxorubicin toxicity in mice [31, 32]. However, other authors have observed unchanged systolic function two days after doxorubicin treatment [8]. These different results are probably related to differences in doxorubicin dose (25 mg vs 20 mg), evaluation periods, and animal models (mice vs rats) [10, 31].

The isolated heart study revealed increased passive myocardial stiffness in the Doxo group, characterized by lower strain under 20 g/cm² diastolic stress (Figure 1). Diastolic
and systolic functional parameters did not differ between groups. *In vitro* cardiac function is dependent on intrinsic myocardial properties and ventricular remodeling and is not influenced by hemodynamic condition. Preserved *in vitro* systolic function found in this study suggests that *in vivo* systolic dysfunction was probably related to changes in hemodynamic conditions such as decreased intravascular volume and increased afterload. Dehydration and decreased intravascular volume was suggested by lower final body weight in the Doxo group [30]. Increased afterload could have been caused by vasoconstriction due to post-doxorubicin dehydration and neurohormonal activation.

The physiopathological mechanisms involved in cardiac dysfunction and increased myocardial stiffness are not completely understood. Several factors could be involved, including interstitial collagen deposition, myocardial edema, and muscle relaxation impairment caused by calcium homeostasis changes. Fibrosis was not expected in this model due to the early evaluation period. In fact, interstitial collagen volume fraction did not differ between groups.

MMP are synthesized as inactive zymogens, which are activated when the propeptide domain is cleaved. MMP are involved in the degradation of extracellular matrix components and are essential for normal tissue remodeling and growth process. MMP-2 and MMP-9, also known as gelatinases, have a high affinity for fibrillar collagen, type IV collagen, fibronectin, and laminin. MMP are present in normal myocardium; however, during cardiac injury such as ischemia, pressure or volume overload, and toxic injury, MMP become over activated [11]. Studies have shown that ventricular dilation is related to MMP activation [33-36]. In this study, the Doxo group presented increased MMP-2 activity with preserved LV diastolic diameter. It is therefore probable that MMP-2 activation precedes LV dilation. Additional studies over different time periods are needed to confirm this hypothesis.

Intracellular MMP-2 activation can also occur during increased oxidative stress [37, 38]. In cardiac myocyte, MMP-2 activation is involved in the degradation of sarcomeric and cytoskeletal proteins such as troponin I, alpha-actinin, and myosin light chain, inducing acute contractile dysfunction [37]. Doxorubicin has been shown to acutely and chronically increase oxidative stress [39, 40]. Therefore, the intracellular MMP-2 activation in this study could be involved in LV dysfunction.

Chronically, doxorubicin-induced myocardial dysfunction is associated with intracellular calcium handling impairment [41, 42]. Studies have also described increased myocyte calcium concentration and changes in the expression of intracellular calcium transient proteins; these include decreased gene expression of Serca-2a, phospholamban, and ryanodine receptor [43-45]. However, we did not find any studies evaluating the expression of gene encoding proteins which affect calcium homeostasis after acute doxorubicin toxicity. Serca-2a, phospholamban, and ryanodine receptor gene expression was unchanged in our Doxo group. As protein levels and function can also be regulated by posttranscriptional mechanisms, we cannot discard the possible involvement of intracellular calcium transient changes in increased passive myocardial stiffness and left ventricular dysfunction. Furthermore, calcium intracellular concentration can be changed by sarcoplasmic reticulum independent mechanisms [46].

Inflammatory cytokines, specifically IL-1 and TNF-α, can activate MMP and modify extracellular cardiac matrix [11]. Chronically, doxorubicin can increase TNF-α [47] and IL-1 [48] gene expression in rats. However, in this study myocardial TNF-α levels did not change after doxorubicin administration. This agrees with other authors who did not find changes in TNF-α serum and myocardial levels during the early stage of doxorubicin-induced cardiomyopathy [49]. Also, IL-10 did not change after doxorubicin. This is in contrast with studies on acute evaluation in mice or chronic evaluation in rats, which showed increased myocardial levels of IL-10 [50-52].

One limitation of this study is that we evaluated only acute cardiotoxicity. Therefore, additional studies are needed to determine whether acute cardiac changes can predict the pattern of chronic cardiac remodeling and left ventricular dysfunction.
In conclusion, acute doxorubicin administration induces *in vivo* left ventricular dysfunction and increased *in vitro* passive myocardial stiffness in rats. Cardiac dysfunction is combined with myocardial increase in MMP-2 activity. Increased inflammatory stimulation or changed gene expression of the proteins involved in intracellular calcium transient are not involved in acute cardiac dysfunction.

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**Disclosure Statement**

There is no conflict of interest to disclosure

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