

Original Paper

Pamidronate Attenuates Oxidative Stress and Energetic Metabolism Changes but Worsens Functional Outcomes in Acute Doxorubicin-Induced Cardiotoxicity in Rats

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Key Words

Isolated heart study • Left ventricular dysfunction • Matrix metalloproteinase • Hypocalcaemia

Abstract

Background: Cardiotoxicity is the major side effect of doxorubicin. As mechanisms that are involved in cardiotoxicity are ambiguous, new methods for attenuating cardiotoxicity are needed. Recent studies have shown that bisphosphonates can decrease oxidative stress. Therefore, the objective of this study was to evaluate the effect of pamidronate on preventing acute doxorubicin-induced cardiotoxicity. **Methods:** Sixty-four male Wistar rats were allocated into four groups: the control group (C), the pamidronate group (P), the doxorubicin group (D) and the doxorubicin/pamidronate group (DP). The rats in the P and DP groups received pamidronate injections (3 mg/kg, IP). After 24 hours, the rats in the D and DP groups received doxorubicin injections (20 mg/kg, IP). Forty-eight hours after doxorubicin injection, the rats were killed. Echocardiography, isolated heart study and biochemical analysis were performed. **Results:** Doxorubicin-induced acute cardiotoxicity showed increased matrix metalloproteinases (MMP)-2 activation, oxidative damage and induced alterations in myocardial energetic metabolism. Pamidronate did not inhibit MMP-2 activation but attenuated oxidative stress and improved myocardial energetic metabolism. Regarding cardiac function, the DP group exhibited a decrease in the left ventricular ejection fraction in the echocardiography and a decrease in $+dP/dt$ in the isolated heart study compared with other groups. The same DP group presented serum hypocalcaemia. **Conclusions:** Despite its ability to reduce oxidative stress and improve energy metabolism in the heart, pamidronate worsened systolic function in rats treated with doxorubicin, and therefore we cannot recommend its use in conjunction with anthracycline chemotherapy.

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Introduction

Doxorubicin is a drug that is currently used in cancer chemotherapy with significant results, primarily in solid and hematologic tumours. Antineoplastic activity occurs as doxorubicin causes damage to cancer cell DNA [1]. Despite its high effectiveness, doxorubicin causes serious side effects, such as cardiotoxicity [2].

Although clinical manifestations appeared weeks after treatment, myocardial aggression began immediately after doxorubicin injection, with acute left ventricular dysfunction after doxorubicin injection [3-5]. Some mechanisms are simultaneously involved in cardiotoxicity that is induced by doxorubicin, such as matrix metalloproteinases (MMP)-2 activation [4, 6], and an increase in oxidative stress [7, 8].

MMPs are members of a protease family that is responsible for extracellular and intracellular matrices degradation [9]. These enzymes can be activated in several ways, such as mechanical and chemical stimuli [10]. They are involved in regular tissue growth and in pathological conditions, such as doxorubicin-induced cardiotoxicity, in which occurs an increase in myocardial MMP gene expression [11] and myocardial MMP activity [4, 6], primarily MMP-2.

One of the most important factors for MMP activation is alterations in oxidative status. Doxorubicin increases myocardial lipid peroxidation and decreases glutathione peroxidase levels early in doxorubicin cardiotoxicity [12, 13]. Myocardium is more susceptible to oxidative damage than other tissues as it contains low levels of catalase and glutathione peroxidase. These enzymes can be rapidly inactivated in the presence of doxorubicin [14].

In addition, changes in myocardial energetic metabolism have a crucial role in cardiac remodelling. When myocardium is subjected to aggression, the main energy supply changes and myocardium increases glucose metabolism and decreases lipid metabolism to produce additional ATP with less oxygen waste [15]. However, ATP production in mitochondria is an important source of reactive oxygen species generation [16].

Bisphosphonates are drugs that are employed in the treatment of bone diseases, such as osteoporosis and osteolytic metastasis [17]. These drugs are frequently combined with cancer chemotherapy to treat neoplastic complications. Several *in vivo* and *in vitro* studies showed an improvement in doxorubicin antitumor activity when zoledronic acid was given [18, 19]. Few studies have addressed the role of bisphosphonates in preventing side effects of doxorubicin treatment.

The recent use of bisphosphonates was associated with decreased MMP activation [20-22]. In addition, bisphosphonates treatment in patients with several types of cancer decreased serum lipid hydroperoxide levels and increased serum glutathione peroxidase levels [23].

Therefore, we hypothesized that pamidronate, which is a type of bisphosphonate, can attenuate acute doxorubicin-induced cardiotoxicity due to pamidronate role in MMP-2 activation and oxidative balance.

Materials and Methods

Study design

This study was approved by Botucatu Medical School-UNESP ethics committee (protocol number 997/2013) and experiments were performed conform the National Institute of Health guidelines. The number of animals was determined by sample size calculations based on previous results of left ventricular fractional shortening [4]. We employed 64 male Wistar rats, whose body weight ranged between 250 and 350 g. The animals were kept in a controlled environment (temperature $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$, dark-light 12-hour period, and water and food ad libitum). Food and water ingestion were measured during the experiment (a knowing amount of water and food were offered and in the end of the day, we measured the leftovers).

The rats were allocated in four groups: the control group (C), the doxorubicin group (D), the pamidronate group (P) and the doxorubicin/pamidronate group (DP). Prior to the experiment, all animals

were submitted to echocardiography. The rats allocated in the P and DP groups received a single dose of pamidronate (3 mg/kg, IP) and the C and D groups received sterile saline injections. Twenty-four hours after pamidronate injection, the rats allocated in the D and DP groups received a single dose of doxorubicin (20 mg/kg, IP), and the C and P groups received sterile saline injections.

Forty-eight hours after doxorubicin injection, we performed a new echocardiography. Immediately after echocardiography, the rats were anesthetized with thiopental (80 mg/kg, IP) to perform an isolated heart study (eight animals per group). The hearts that were submitted to the isolated heart study were not utilized for any other analysis due to retrograde perfusion can be interfered in a posterior biochemical analysis. Other animals (eight animals per group) were submitted to anaesthesia with thiopental, and submitted to euthanasia by heart excision. Hearts were washed with sterile saline and cardiac tissues were frozen at -80°C . Blood was collected by left ventricle puncture, centrifuged and serum was stored at -80°C .

Doxorubicin administered for rats at the doses of 20 mg/kg is a suitable model to study the side effects of doxorubicin in cardiac tissue [3, 4, 11, 24]. This dose in rats is equivalent to 250 mg in a 70 kg human [25]. The pamidronate infusion dose was based on previous studies with small animals, which demonstrated the pharmacological effect of the drug [26, 27], and is equivalent to 35 mg in a 70 kg human [25].

Echocardiography

All animals were submitted to echocardiographic evaluation prior to the study and 48 hours after doxorubicin injection. The rats received a light anaesthesia with ketamine (50 mg/kg, IP) and xylazine (1 mg/kg, IP). Echocardiography was performed by the same examiner using Vivid S6 (General Electric Medical Systems) equipment with a multifrequency transducer in the range of 5 to 11.5 MHz. The examiner was blinded to the groups.

Parameters were obtained from the parasternal long and short axes and apical four chambers. The analysed structural variables included the left atrial diameter, the left ventricular diastolic posterior wall thickness, and the left ventricular diastolic and systolic diameters. Diastolic function was evaluated by the transmitral Doppler E wave, the A wave, the E/A waves ratio, and the isovolumetric relaxation time. Systolic function was analysed by the ejection fraction and LV fractional shortening [28].

Isolated Heart Study: Langendorff preparation

The rats were anesthetized with thiopental (80 mg/kg, IP) and received unfractionated heparin (2000 I.U., IP). After sternotomy, the rats were artificially ventilated, and the ascending aorta was dissected and cannulated. Retrograde perfusion was initiated with a modified Krebs-Henseleit solution (NaCl 118.5 mmol/L; KCl 4.69 mmol/L; CaCl_2 2.52 mmol/L; MgSO_4 1.16 mmol/L; KH_2PO_4 1.18 mmol/L; glucose 5.5 mmol/L; NaHCO_3 25.88 mmol/L; and mannitol 8 mmol/L) at 37°C , constantly gassed with a mixture of 95% O_2 and 5% CO_2 , and perfused pressure 75 mmHg [4]. The heart was removed and transferred to an isolated perfused heart apparatus (size 3, type 830, Hugo Sachs Elektronik - March-Hugstetten, Germany). A balloon was inserted in the left ventricular cavity, and the volume inside the balloon was modified to obtain a diastolic pressure of zero at 25 mmHg. We registered the diastolic and systolic pressures, the maximum left ventricular pressure decrease rate ($-\text{dP}/\text{dt}$) and the maximum left ventricular pressure development rate ($+\text{dP}/\text{dt}$). We calculated the stress (g/cm^2) and strain (%) with previously described formulas [4, 29].

Systolic function was evaluated by $+\text{dP}/\text{dt}$, and the maximum systolic pressure was obtained. Diastolic function was analysed by $-\text{dP}/\text{dt}$. Diastolic pressure-volume and diastolic stress-strain relationship curves were constructed, and we calculated curves inclination and area under curves to compare the groups. We evaluated the left ventricle compliance by the diastolic pressure-volume relationship and myocardial passive stiffness by the diastolic stress-strain relationship.

Zymography: MMP-2 evaluation

Zymography was performed as previously described method [4]. Briefly, thirty milligrams of cardiac tissue (apex of left ventricle) were added to an extraction buffer, crushed and centrifuged; supernatant was collected. Proteins were quantified by the Bradford method. The samples (20 μg of protein for each sample) were diluted in the sample buffer. Electrophoresis was performed in an 8% polyacrylamide and 1% gelatine gel in the Bio-Rad mini-protean system. After running, the gels were washed with 2.5% Triton X-100 and with Tris-HCl 50 mM, pH 8.4. The gels were incubated for 16 hours at 37°C with continued agitation. The gels were stained by 2.5% Coomassie Brilliant blue and after discoloured. The same control sample was

included in each gel to normalize the results. To identify MMP-2, we employed a recombinant rat/mouse MMP-2 standard (R&D Systems). The gels were photographed by Gel Logic 6000 Pro (Carestream Health Inc.), and the intensity of the gelatinolytic action was analysed by GelPro 3.1.

Oxidative stress and energetic metabolism

Heart tissue (200 mg) was added to a 0.01 M sodium phosphate buffer (pH 7.4), centrifuged for 30 minutes (12000 rpm, at -4°C) and protein-quantified by the Bradford method in a supernatant. Measurements were performed by a Pharmacia Biotech spectrophotometer (UV/visible Ultrospec 5000 with Swift II Application software to computer system control, 974213, Cambridge, England, UK) at 560 nm. A supernatant was used to determine lipid hydroperoxide concentrations, antioxidant and metabolic enzyme activity. A pellet was used to determine mitochondrial respiratory chain enzymatic complex activities.

The lipid hydroperoxide concentration in myocardial was employed as an oxidation marker. Myocardial activity of the antioxidant enzymes catalase (E.C.1.11.1.6.), superoxide dismutase (E.C.1.15.1.1.) and glutathione peroxidase (E.C.1.11.1.9.) were evaluated. The concentrations were determined according to previously described methods [30, 31].

Regarding energetic metabolism, we evaluated phosphofructokinase, β -hydroxyacyl dehydrogenase, lactate dehydrogenase, pyruvate dehydrogenase and citrate synthase activity [32]. Similarly, we measured activities of mitochondrial respiratory chain enzymatic complex I, complex II and ATP synthase [33].

Serum calcium

Measurement was conducted using a Bioclin kit (reference number K051 - Quibasa Quimica Basica Ltda, MG, Brazil) by colorimetric reaction of calcium with arzenazo III, which produced a blue colour, evaluated in 600 and 680 nm wavelengths.

Statistical analysis

Variables were expressed as the mean $\pm$ standard deviation. Variables with a non-normal distribution were transformed for normalization. Comparisons were conducted by two-way ANOVA. When we observed an interaction between the two factors, we performed comparisons among the groups of interest. When we did not observe an interaction, we showed isolated factor data. We assumed a statistically significant level of 5% for all analyses. We were not able to normalize serum calcium values; therefore, serum calcium comparisons were performed by the t-test or the Mann-Whitney test. In this situation, the p value was adjusted by Bonferroni correction, which p value was multiplied by the number of comparisons performed (6 comparisons).

Results

Body weight, food and water ingestion

Body weight was similar in all groups prior to treatment. After drug injection, the rats that received doxorubicin exhibited decreased body weight. These same animals exhibited decreased water and food ingestion. Left ventricle weight did not differ among the groups, even when the values were corrected by body weight. Pamidronate did not change these parameters. These results are exhibit in Table 1.

Echocardiography

We performed an echocardiographic study prior to the experiment; the results for all groups were similar (data not shown). The echocardiographic parameters after treatment are listed in Table 2. Doxorubicin treatment caused a decrease in the left atrium diameter and the left ventricular diastolic diameter and an increase in the left ventricular posterior wall relative thickness (PWRT). In the DP group we observed a decrease in the PWRT compared with the D group.

The DP group had a smaller left ventricular ejection fraction and left ventricular fractional shortening than the D group and the P group, which indicated poor systolic function when the drugs were simultaneously administered.

Table 1. Body weight, food and water ingestion and left ventricular weight. C: control group; P: pamidronate group; D: doxorubicin group; DP: doxorubicin + pamidronate group. BW: body weight; LVW: left ventricle weight; BW initial represents the BW before doxorubicin injection. BW final represents the BW 48 hours after doxorubicin injection. Food and water intake were quantified for 48 hours after doxorubicin injection. Values are expressed in mean $\pm$ standard deviation. p: two-way ANOVA. p_i : p value of interaction; p_d : p value of doxorubicin; p_p : p value of pamidronate

	C (n=16)	P (n=16)	D (n=16)	DP (n=16)	p_i	p_d	p_p
BW initial (g)	333 $\pm$ 16.8	317 $\pm$ 30.3	331 $\pm$ 26.9	337 $\pm$ 37.8	0.143	0.252	0.498
BW final (g)	338 $\pm$ 21.4	323 $\pm$ 33.2	305 $\pm$ 26.1	316 $\pm$ 34.6	0.086	0.008	0.782
Water intake (mL)	113 $\pm$ 18.3	111 $\pm$ 19.8	74.7 $\pm$ 21.4	75.6 $\pm$ 17.1	0.756	<0.001	0.906
Food intake (g)	72.2 $\pm$ 9.54	67.6 $\pm$ 8.41	33.7 $\pm$ 10.8	31.1 $\pm$ 13.7	0.721	<0.001	0.188
LVW (g)	0.80 $\pm$ 0.11	0.75 $\pm$ 0.11	0.71 $\pm$ 0.11	0.73 $\pm$ 0.14	0.251	0.074	0.562
LVW/BW (g/kg)	2.37 $\pm$ 0.26	2.33 $\pm$ 0.26	2.35 $\pm$ 0.33	2.31 $\pm$ 0.30	0.999	0.773	0.607

Table 2. Echocardiography 48 hours after doxorubicin injection. HR: heart rate in beats per minute. LA: left atrium diameter; LVDD: left ventricular diastolic diameter; BW: body weight; LVSD: left ventricular systolic diameter; PWRT: left ventricular posterior wall relative thickness (2xposterior wall thickness/LVDD); E/A: E and A wave ratio, when E wave represents the peak velocity of early ventricular filling and A wave represents the peak velocity of transmitral flow during atrial contraction; IVRTc: isovolumetric relaxation time corrected by heart rate. LVEF: left ventricular ejection fraction; LVFS: left ventricular fractional shortening. C: control group; P: pamidronate group; D: doxorubicin group; DP: doxorubicin + pamidronate group. Values are expressed in mean $\pm$ standard deviation. P value: two-way ANOVA. p_i : p value of interaction; p_d : p value of doxorubicin; p_p : p value of pamidronate. When we observed interaction between factors (p_i <0.05), we performed comparison between groups. #: show the difference between control group; &: show the difference between doxorubicin group; §: show the difference between pamidronate group

	C (n=15)	P (n=16)	D (n=15)	DP (n=16)	p_i	p_d	p_p
HR (bpm)	287 $\pm$ 46.0	294 $\pm$ 29.2	296 $\pm$ 45.1	274 $\pm$ 53.5	0.195	0.603	0.195
LA (mm)	1.41 $\pm$ 0.11	1.32 $\pm$ 0.11#	1.31 $\pm$ 0.12#	1.41 $\pm$ 0.11&§	0.001	0.969	0.814
LVDD (mm)	7.15 $\pm$ 0.72	7.14 $\pm$ 0.54	6.20 $\pm$ 0.44	6.77 $\pm$ 0.69	0.069	<0.001	0.072
LVDD/BW (mm/kg)	21.2 $\pm$ 1.68	22.2 $\pm$ 1.79	20.5 $\pm$ 2.28	21.7 $\pm$ 2.95	0.930	0.266	0.053
LVSD (mm)	3.35 $\pm$ 0.64	3.33 $\pm$ 0.42	2.96 $\pm$ 0.54	3.68 $\pm$ 0.58&	0.011	0.896	0.016
PWRT	0.37 $\pm$ 0.07	0.36 $\pm$ 0.06	0.51 $\pm$ 0.07#	0.41 $\pm$ 0.09&	0.018	<0.001	0.009
E/A	1.40 $\pm$ 0.31	1.36 $\pm$ 0.27	1.57 $\pm$ 0.45	1.46 $\pm$ 0.25	0.701	0.114	0.388
IVRTc	56.0 $\pm$ 10.7	62.3 $\pm$ 16.0	57.0 $\pm$ 14.5	52.9 $\pm$ 13.6	0.169	0.260	0.774
LVEF	0.89 $\pm$ 0.05	0.90 $\pm$ 0.03	0.88 $\pm$ 0.06	0.83 $\pm$ 0.06&§	0.035	0.007	0.077
LVFS	0.53 $\pm$ 0.07	0.53 $\pm$ 0.05	0.52 $\pm$ 0.08	0.46 $\pm$ 0.06&§	0.048	0.012	0.061

Table 3. Isolated heart study. C: control group; P: pamidronate group; D: doxorubicin group; DP: doxorubicin + pamidronate group. V_0 : volume insert in latex balloon inside left ventricle to achieve a diastolic pressure of zero; $-dP/dt$: the maximum left ventricular pressure decrease rate; $+dP/dt$: the maximum left ventricular pressure development rate; SP_{max} : the maximum systolic pressure obtained. Values are expressed in mean $\pm$ standard deviation. p: two-way ANOVA. p_i : p value of interaction; p_d : p value of doxorubicin; p_p : p value of pamidronate. *Values were mathematically transformed for statistical analysis

	C (n=8)	P (n=8)	D (n=8)	DP (n=8)	p_i	p_d	p_p
V_0 (μ L)	90 $\pm$ 19	89 $\pm$ 31	100 $\pm$ 32	88 $\pm$ 23	0.580	0.699	0.535
$-dP/dt$ (mmHg/s)	1825 $\pm$ 259	2125 $\pm$ 204	1734 $\pm$ 561	1547 $\pm$ 258	0.095	0.025	0.692
$+dP/dt$ (mmHg/s)	2550 $\pm$ 381	2982 $\pm$ 183	2516 $\pm$ 809	2125 $\pm$ 395	0.050	0.035	0.918
SP_{max} (mmHg)*	116 $\pm$ 7.8	132 $\pm$ 9.3	114 $\pm$ 29	105 $\pm$ 14	0.157	0.042	0.630

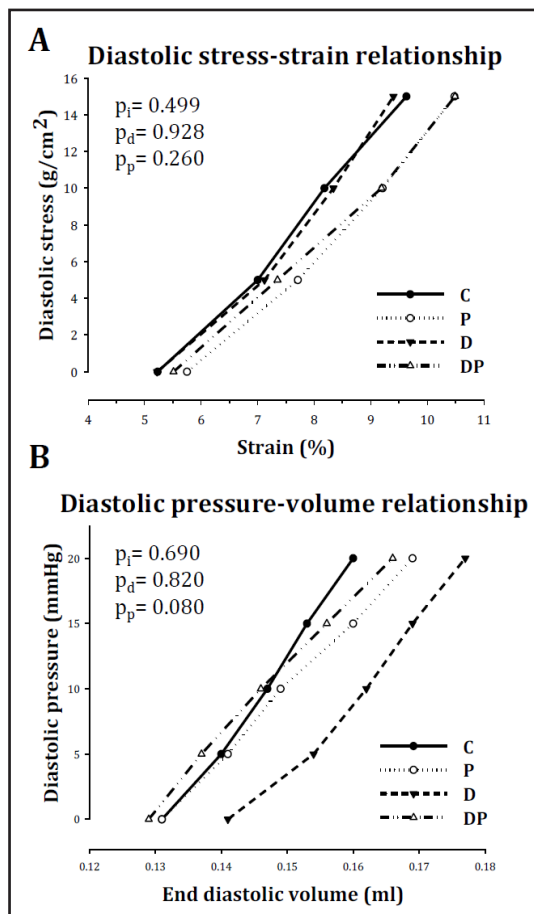


Fig. 1. Panel A: Diastolic stress-strain relationship represents an index of myocardial passive stiffness. Panel B: Diastolic pressure-volume relationship represents an index of left ventricle compliance. The curves inclinations and area under curves were calculated; they did not differ between groups in both relationships; p value: two-way ANOVA; n=8 animals/group.

We did not observe differences in diastolic function as the isovolumetric relaxation time corrected by heart rate and E/A waves relation were similar between the groups.

Isolated perfused heart study

The isolated heart study results

are shown in Table 3. The initial intraventricular balloon volume was similar among the groups. The rats that were treated with doxorubicin had diastolic dysfunction as indicated by a lower maximum left ventricular pressure decrease rate ($-\text{dP}/\text{dt}$). These animals had lower maximum systolic pressure than rats that did not receive doxorubicin. Pamidronate did not change these results. The maximum LV pressure development rate ($+\text{dP}/\text{dt}$), which represents systolic function, of the DP group was worse than $+\text{dP}/\text{dt}$ for other groups. The diastolic pressure-volume and diastolic stress-strain relationships did not differ among animals, as shown in Figure 1.

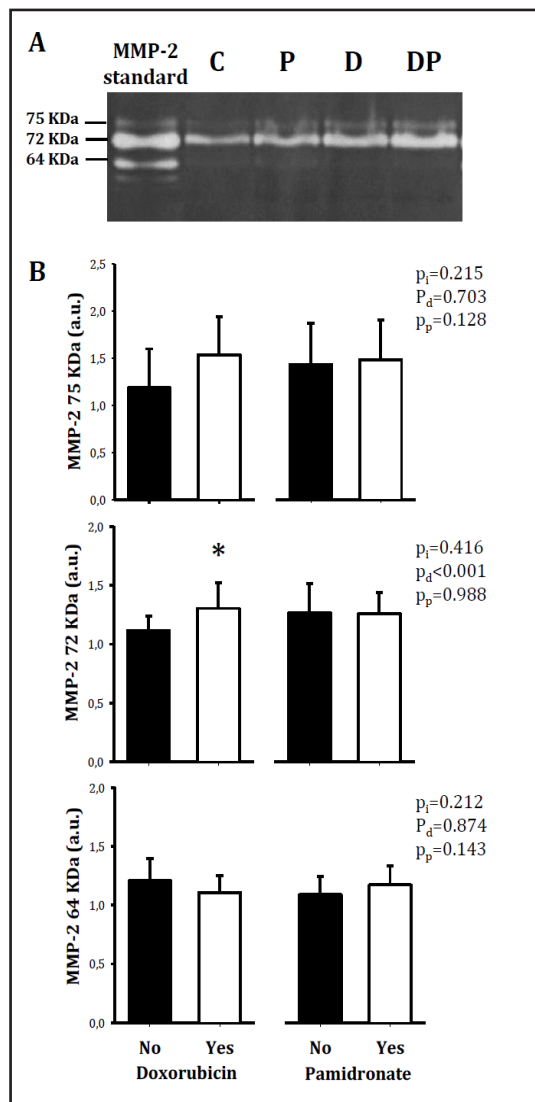


Fig. 2. Matrix metalloproteinase (MMP)-2 activation in myocardial tissue. Panel A: zymography gel; white bands correspond to gelatine degradation. Panel B: 75 KDa, 72 KDa and 64 KDa MMP-2 activity. C: control group; P: pamidronate group; D: doxorubicin group; DP: doxorubicin + pamidronate group. Symbol * indicates significant statistical difference. Statistical analysis: two-way ANOVA; p_i : p value of interaction; p_d : p value of doxorubicin; p_p : p value of pamidronate.

Table 4. Myocardial oxidative stress. C: control group; P: pamidronate group; D: doxorubicin group; DP: doxorubicin + pamidronate group. Values are expressed in mean $\pm$ standard deviation. p: two-way ANOVA. p_i : p value of interaction; p_d : p value of doxorubicin; p_p : p value of pamidronate. *Values were mathematically transformed for statistical analysis. When we observed interaction between factors ($p_i < 0.05$), we performed comparison between groups. #: show the difference between control group; &: show the difference between doxorubicin group; §: show the difference between pamidronate group

	C (n=8)	P (n=7)	D (n=6)	DP (n=7)	P_i	P_d	P_p
Lipid hydroperoxide (nmol/g of tissue)	185 $\pm$ 23	175 $\pm$ 26	236 $\pm$ 26#	181 $\pm$ 27&	0.042	0.010	0.004
Catalase* (μ mol/g of tissue)	55.4 $\pm$ 6.66	46.2 $\pm$ 5.65#	37.4 $\pm$ 4.16#	54.5 $\pm$ 9.04&§	<0.001	0.043	0.097
Superoxide dismutase (nmol/g of tissue)	19.3 $\pm$ 3.34	15.9 $\pm$ 1.89#	11.2 $\pm$ 1.71#	17.0 $\pm$ 2.25&	<0.001	0.002	0.243
Glutathione peroxidase (nmol/g of tissue)	26.7 $\pm$ 4.69	38.9 $\pm$ 7.97	14.0 $\pm$ 3.83	28.1 $\pm$ 5.41	0.688	<0.001	<0.001

Table 5. Myocardial energetic metabolism. C: control group; P: pamidronate group; D: doxorubicin group; DP: doxorubicin + pamidronate group. Values are expressed in mean $\pm$ standard deviation. p: two-way ANOVA. p_i : p value of interaction; p_d : p value of doxorubicin; p_p : p value of pamidronate. *Values were mathematically transformed for statistical analysis. When we observed interaction between factors ($p_i < 0.05$), we performed comparison between groups. #: show the difference between control group; &: show the difference between doxorubicin group; §: show the difference between pamidronate group

	C (n=8)	P (n=7)	D (n=6)	DP (n=7)	P_i	P_d	P_p
Phosphofructokinase (nmol/mg of tissue)	0.90 $\pm$ 0.27	1.50 $\pm$ 0.32#	1.87 $\pm$ 0.29#	1.67 $\pm$ 0.59	0.021	0.001	0.220
β -hydroxyacyl Co-A dehydrogenase (nmol/mg of tissue)	20.9 $\pm$ 1.98	23.5 $\pm$ 2.73#	10.6 $\pm$ 1.43#	22.4 $\pm$ 1.91&	<0.001	<0.001	<0.001
Lactate dehydrogenase* (nmol/mg of tissue)	3.52 $\pm$ 0.24	4.11 $\pm$ 0.30#	7.70 $\pm$ 1.29#	4.81 $\pm$ 0.74&§	<0.001	<0.001	0.003
Pyruvate dehydrogenase (nmol/mg of tissue)	1.04 $\pm$ 0.20	1.22 $\pm$ 0.16	1.03 $\pm$ 0.13	1.20 $\pm$ 0.24	0.911	0.870	0.034
Citrate synthase* (nmol/mg of tissue)	48.2 $\pm$ 8.04	109 $\pm$ 17.1#	29.1 $\pm$ 5.19#	95.3 $\pm$ 11.1&	0.006	<0.001	<0.001
Complex I (nmol/mg of tissue)	44.1 $\pm$ 5.68	61.5 $\pm$ 9.24	34.2 $\pm$ 4.04	41.0 $\pm$ 5.23	0.054	<0.001	<0.001
Complex II (nmol/mg of tissue)	9.22 $\pm$ 1.86	6.59 $\pm$ 0.92#	4.81 $\pm$ 1.13#	6.48 $\pm$ 1.39	<0.001	<0.001	0.412
ATP synthase (nmol/mg of tissue)	25.2 $\pm$ 2.61	25.9 $\pm$ 3.38	16.6 $\pm$ 1.85#	23.1 $\pm$ 4.52&	0.040	<0.001	0.012

Zymography

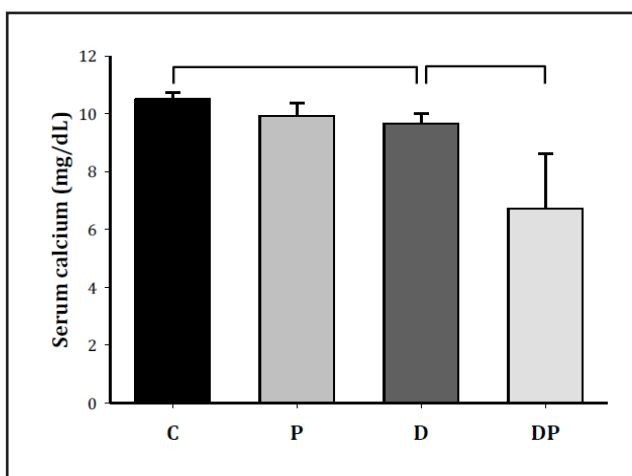
We performed zymography in eight samples for each group. The same control sample was inserted in each gel to normalize the results. We employed a recombinant mouse/rat MMP-2 standard to identify MMP-2 in gels. We identified three bands that correspond to MMP-2; their molecular weights were 75 KDa, 72 KDa and 64 KDa [34]. We observed an increase in 72 KDa MMP-2 in animals that received doxorubicin. We did not observe the effect of pamidronate in MMP-2 activity. Zymography results are shown in Figure 2.

Myocardial oxidative stress

Doxorubicin treatment increased oxidative stress as shown by an increase in myocardial lipid hydroperoxide concentration. When rats were also treated with the pamidronate (DP group), myocardial lipid hydroperoxide concentration became similar to the concentration of the control group, as shown in Table 4.

Myocardial superoxide dismutase and catalase activities decreased in the doxorubicin and pamidronate group; however, when the drugs were simultaneously administered, the enzyme activities increased and remain similar to the control group levels.

Fig. 3. Figure shows serum calcium concentration. C: control group (n=8); P: pamidronate group (n=8); D: doxorubicin group (n=8); DP: doxorubicin + pamidronate group (n=8). Following comparisons were performed: C vs P; C vs D; D vs DP; and P vs DP. Statistical analysis: Mann-Whitney test; p value obtained was adjusted (multiplied by 6); we considered p value significant if was <0.05 after correction. Brackets link groups statistically different.



Myocardial glutathione peroxidase activity increased in animals that received pamidronate and decreased in animals that received doxorubicin; however, an interaction between the two treatments in glutathione peroxidase levels was not observed.

Myocardial energetic metabolism

We observed a decrease in myocardial β -hydroxyacyl Co-A dehydrogenase, citrate synthase; complex II and ATP synthase activities in the D group when compared with the C group. We observed an increase in phosphofructokinase and lactate dehydrogenase activities for the same group. When we infused doxorubicin and pamidronate (DP group), citrate synthase and β -hydroxyacyl Co-A dehydrogenase activities were higher than levels in the D group, and ATP synthase activities increased to C group levels. In the same manner, the DP group showed smaller lactate dehydrogenase myocardial activities than activities in the D group. These results are shown in Table 5.

Serum calcium

The D group showed lower levels of serum calcium than the C group levels, whereas the P group and C group yielded similar values. When the rats received both drugs, serum calcium was significantly decreased compared with the D group, as shown in Figure 3.

Discussion

Bisphosphonate, including pamidronate, has been tested in off label situations to prevent inflammation, cellular proliferation and oxidative damage, for instance, in rheumatologic diseases. Its use as a chemotherapy agent has been investigated with acceptable results; however, studies have not addressed the role of bisphosphonates in prevention chemotherapy side effects. In this study, we evaluated the effect of pamidronate in acute doxorubicin-induced cardiotoxicity prevention. We showed that doxorubicin induced acute cardiotoxicity, with an increase in myocardial MMP-2 activation, oxidative stress and changes in energy metabolism. Pamidronate decreased oxidative damage and improved the activity of energetic metabolism enzymes; however, it did not prevent MMP activation. LV systolic function worsened by an unidentified mechanism when doxorubicin and pamidronate were simultaneously administered.

Regarding signals of general doxorubicin toxicity, we observed a decrease in body weight in animals that received doxorubicin due to a decrease in water and feed intake, as shown in previous studies [5, 35]. A decrease in water intake can cause dehydration and may explain the reduction in the left atrium and left ventricular diastolic diameter and the increase in the PWRT, as depicted by the echocardiogram. This situation, which is referred

to as pseudo hypertrophy [36], can be attributed to dehydration. However, the rats in the DP group exhibited the same reduction in water and feed intake, which was associated with the lack of differences in the left atrium and diastolic diameters compared with animals in the D group. In this group, we observed a lower PWRT than the thickness measured for the D group. Therefore, we hypothesized that an increase in the PWRT in the D group was caused by myocarditis, which was induced by doxorubicin [37].

In addition to various types of heart disease, we observed that doxorubicin-induced cardiotoxicity causes changes in cellular energy supply. In pathological situations, a change in substrate preferences occurs and the main substrate becomes the glucose, which is associated with mitochondrial dysfunction [38]. When doxorubicin was administered in previous studies, a decrease in ATP generation [39] and changes in lipid and glucose cellular metabolism [40] were observed. We observed an increase in phosphofructokinase myocardial activity due to an increase in glucose metabolism and a decreased in β -hydroxyacyl CoA dehydrogenase activity and a consequent decrease in lipid oxidation.

We also showed that doxorubicin-treated hearts present lower mitochondrial enzymatic complex activity (complex I, complex II and ATP synthase) compared with the control animals. When pamidronate was simultaneously administered (DP group), ATP synthase activity was improved to levels of the C group. If there is an impairment of mitochondrial electron transport chain, an accumulation of unpaired electrons, reactive oxygen species generation and a consequent increase in cellular oxidative stress occurs. An important source of reactive oxygen species is mitochondrial ATP generation.

Considering the effect of doxorubicin in the redox status, the first defence against oxidative damage is provided by the antioxidants system, primarily the enzymatic system. In this study, we observed a decrease in myocardium catalase and superoxide dismutase activity in the D group compared with the C group, which was diminished by pamidronate. These results indicate that myocardium probably spent a large amount of antioxidants enzymes to modulate increases in oxidative stress in the D group. However, this excessive consumption was not necessary when pamidronate was simultaneous administered. We demonstrated that doxorubicin increased oxidative damage. In doxorubicin-induced cardiotoxicity, polyunsaturated fatty acids in the cell membranes are damaged and cause cell injury and generated lipid hydroperoxide and other products [41]. Another relevant result of this study was that pamidronate prevented an increase in myocardial lipid hydroperoxide levels that were induced by doxorubicin. Therefore, our data strongly suggest that pamidronate attenuated cardiac oxidative stress that was induced by doxorubicin.

Reactive oxygen-nitrogen species generation is an important MMP activator [42]. When the tissues are subjected to mechanical or chemical stress, these enzymes are activated and are responsible for extracellular matrix degradation. As showed previously by other studies [4, 6, 11], we observed MMP-2 activation and no effect of pamidronate in this result. Several studies showed inhibit MMP-2 activation when bisphosphonates were used [20-22]; however, Gonçalves et al [43] indicated that pamidronate did not affect MMP activation.

Regarding the side effects of bisphosphonates, serum calcium can be quantified as pamidronate can induce hypocalcaemia [44]. However, we observed hypocalcaemia in the D group and more pronounced in the DP group. The reason that doxorubicin-induced hypocalcaemia may be associated with doxorubicin-induced hypomagnesaemia and diminished organ responsiveness to PTH [45, 46]. Also, anthracyclines have the ability to chelate divalent cations [47], such as calcium.

Changes in serum calcium may directly interfere in cardiac function as calcium concentration is one of the most important determinants of cardiac contractility. In the echocardiogram that we observed for the same DP group that exhibited hypocalcaemia, impairment in left ventricular systolic function can be explained by hypocalcaemia. However, the same results were observed when we analysed cardiac function *in vitro* by an isolated heart study. In the isolated heart study, hearts were perfused with Krebs-Henseleit solution, which contains a stable calcium concentration. Therefore, hypocalcaemia was not the only reason for systolic dysfunction.

Another important aspect to consider is that some studies have shown a beneficial anticancer effect of bisphosphonates [48, 49], which had us consider that the combined use of doxorubicin and pamidronate may produce better efficacy in tumour treatment and can mitigate the side effects caused by doxorubicin. Thus, studies of the effect of doxorubicin, which are associated with pamidronate, are critical. In our study, pamidronate attenuated the deleterious effects of doxorubicin in energetic metabolism and oxidative stress. However, the beneficial biochemical alterations did not improve cardiac function or promote significant changes in cardiac morphology. A potential explanation is that we employed a short and acute model to study mechanisms that are involved in cardiac toxicity and the required amount of time to change cardiac shape or function was insufficient. A dose-response study with pamidronate may reveal other cardiac outcomes.

In conclusion, acute doxorubicin administration causes left ventricular dysfunction, MMP-2 activation, an increase in oxidative damage, a decrease in myocardial lipid metabolism and an increase in glucose metabolism, which are associated with mitochondrial dysfunction. Combined pamidronate administration decreases oxidative stress and attenuates changes in energetic metabolism, which are associated with poor LV systolic function in this acute model.

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Disclosure Statement

None declared.

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