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Association of the time course of pulmonary arterial hypertension with changes in oxidative stress in the left ventricle

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SUMMARY

1. This study investigates the time course of pulmonary arterial hypertension (PAH) due to monocrotaline (MCT) and its association with cardiac function and oxidative stress markers in the left ventricle (LV).

2. Male Wistar rats were divided into six groups: 7 days, 21 days, and 31 days for both control and MCT groups. Following echocardiographic analysis, the heart was removed. The LV was separated and homogenized to analyze oxidized-to-total glutathione ratio and thioredoxin reductase (TrxR) activity as well as hydrogen peroxide (H_2O_2) and ascorbic acid levels.

3. There was significant ($P < 0.01$) cardiac and right ventricle (RV) hypertrophy and pulmonary congestion in the MCT 21 day and 31 day groups. Echocardiography showed a change in the flow wave of the pulmonary artery at 21 days after MCT treatment. There was an increase in the LV ejection time ($P < 0.05$) at 31 days after MCT. The LV H_2O_2 concentration was increased ($P < 0.05$) in the MCT 21 day and MCT 31 day groups compared with controls. There was a reduction ($P < 0.05$) in the LV ascorbic acid concentration and an increase ($P < 0.05$) in TrxR activity in the MCT 31 day rats.

4. Our findings showed RV changes due to pulmonary hypertension at 21 days after MCT injection. There was a correlation between the degree of dysfunction and the morphometry of the heart chambers, along with impairment of the antioxidant/pro-oxidant balance in the LV 31 days after the beginning of the protocol. This study suggests that LV changes follow RV dysfunction subsequent to pulmonary hypertension.

Key words: ascorbic acid, Cor pulmonale, glutathione, monocrotaline, right heart failure, thioredoxin reductase.

INTRODUCTION

Pulmonary arterial hypertension (PAH) is the diagnosis given to patients with pulmonary hypertension of unknown aetiologies. Patients with PAH present signs and symptoms of right heart failure, and the prognosis has been recognized as being extremely poor. Although the precise aetiology of PAH remains unknown, the findings of increased pulmonary vascular reactivity and vasoconstrictive tendency underlie the development of PAH.[1]

The impaired cardiac function under these circumstances has been generally attributed to right ventricle (RV) dysfunction. However, the left ventricle (LV) may be involved as well because neurohormonal compensation of depressed cardiac function[2] will typically affect both ventricles. Two distinct forms of mechanical interaction exist: a series interaction because RV output equals LV input,[3] and a direct local interaction in which forces are transmitted over the septum or via the pericardium and are independent of humoral or circulatory effects.

Characteristically, echocardiography has been an important tool in the diagnosis, measurement and monitoring of heart disease, particularly as they relate to right ventricular dysfunction, which may or may not be induced by pulmonary hypertension both in experimental medicine and in clinical follow-up for patients.[4,5]

A single injection of monocrotaline (MCT), a pyrrolizidine alkaloid, causes pulmonary arterial hypertension in rats. This model has several pathological features similar to PAH in humans.[6] MCT induces an increase in pulmonary vascular resistance, leading to an enhanced afterload to the RV, which constitutes a stimulus for RV hypertrophy. MCT produces vascular endothelial cell damage, muscular hypertrophy in pulmonary arteries[7] and increased reactive oxygen species (ROS) production in the RV.[8] The extent of pulmonary hypertension as well as RV hypertrophy induced by MCT administration appears to be correlated with these pulmonary vascular structural changes.[9]

The data suggest that oxidative stress, due to an increased ROS production and/or a decrease in antioxidants, is involved in the pathophysiology of pulmonary arterial hypertension (PAH).[10] Farahmand *et al.*[11] provide strong evidence of a close correlation between the status of the myocardial antioxidant and the RV dysfunction induced by MCT. Pichardo *et al.*[7] also found a reduction in the concentration of α-tocopherol, a non-enzymatic antioxidant in this experimental model, which was associated with impairing RV function.

Right ventricular disease has received increasing clinical recognition,[12] and RV dysfunction is the major determinant of survival in patients with PAH, regardless of the underlying cause.[13] RV hypertrophy may progress to heart failure, affecting the function of both cardiac chambers. However, there is a paucity of information in the published reports regarding LV function in PAH not only in clinical but also in experimental studies.

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This study evaluated the progressive development of pulmonary hypertension, leading to RV hypertrophy and failure in rats after MCT administration through echocardiography. Our primary goal was to assess LV function during the transition from hypertrophy to failure by examining oxidative stress changes in this ventricular chamber.

METHODS

Animals and MCT treatment

Forty-two male Wistar rats weighing 160 ± 20 g were used. Experimental animals were housed in a facility at 22–24°C on a 12 : 12 h light : dark cycle, and they had free access to food and water. Animals were weighed weekly to track body weight gain during the experimental protocol. They were also divided into six groups (n = 7/group): three controls at 7, 21 and 31 days (receiving a single i.p. saline solution injection) and three MCT groups at 7, 21 and 31 days (receiving a single i.p. MCT injection at a concentration of 60 mg/kg body weight).[11] The experimental protocol was approved by the Institutional Animal Care and Use Committee. All animals were treated in accordance with the Guidelines for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institute of Health (NIH publication no. 85-23, Revised 1996).

Experimental protocol

At the end of the experimental protocol (7, 21 or 31 days), general anaesthesia was administered by ketamine (90 mg/kg i.p.) and xylazine (10 mg/kg i.p.) injections for echocardiography. Next, hearts and lungs were rapidly excised, weighed, and frozen for posterior analysis. The heart was weighed without atria. After this, the right ventricle free wall was dissected out and weighed. Left ventricle along with the septum was also weighed. The lungs were weighed to estimate the congestion in these organs in terms of tissue weight to body weight ratio. The concentration of hydrogen peroxide was determined using LV slices. LV homogenates were also prepared (1.15% w/v KCl and phenyl methyl sulfonyl fluoride 20 mmol/L) in Ultra-Turrax. This suspension was centrifuged at 1000 g for 10 min at 0–4°C to remove cell debris,[14] and supernatants were used for the assays to determine the oxidized-to-total glutathione ratio (GSSG/GSH) as well as ascorbic acid and thioredoxin reductase activity.

Echocardiography

Animals underwent echocardiography before (baseline evaluation) and after 7, 21 and 31 days of treatment. For echocardiography, the animals were placed in a left lateral decubitus position (45° angle) to obtain cardiac imagery. An EnVisor HD System (Philips Medical, Andover, MA, USA) with a 12–13 MHz transducer was used at a depth of 2 cm using fundamental and harmonic imaging. The images were captured by a trained operator with experience in small animal echocardiography.[15]

Determination of the oxidized-to-total glutathione ratio

To determine the oxidized-to-total glutathione ratio, tissue was deproteinized with 2 mol/L perchloric acid, centrifuged for 10 min at 1000 g, and then the supernatant was neutralized with 2 mol/L potassium hydroxide. The reaction medium contained 100 mmol/L phosphate buffer (pH 7.2), 2 mmol/L nicotinamide dinucleotide phosphate acid, 0.2 U/mL glutathione reductase, and 70 mmol/L 5,5 dithiobis (2-nitrobenzoic acid).[16]

Ascorbic acid concentration

To determine ascorbic acid concentration, samples were incubated for 20 min at 25°C with tungstic acid reagent. This reagent consists of a suspension of sodium tungstate and anhydrous sodium hydrogen phosphate in deionized water. Concentrated sulfuric acid was added slowly, and the suspension was heated to dissolve the sulfuric acid. The solution was heated for 2 h with a reflux condenser to prevent it from boiling, and then, the solution was cooled. This mixture was centrifuged for 10 min at 1000 g. Absorbance values of the supernatant and the vitamin C standard solution (diluted in 50 mmol/L oxalic acid solution) were measured at 700 nm.[17] The results were expressed in μmol.

Thioredoxin reductase activity

Thioredoxin reductase (TrxR) activity was assayed by an *in vitro* reduction of DTNB to 5′-thionitrobenzoic acid (TNB). Briefly, the samples were centrifuged at 1000 g, and 10 μL of supernatant was added to 990 μL of the reaction mix (0.25 mmol/L DTNB, 0.24 mmol/L NADPH, 10 mmol/L EDTA, and 100 mmol/L phosphate buffer, pH 7.5). The conversion of DTNB to TNB was measured spectrophotometrically at 412 nm. Because several enzymes are able to reduce DTNB, a specific TrxR inhibitor was used to determine the reduction of DTNB due only to TrxR activity.[18] These results were expressed in nmol/mg protein.

Hydrogen peroxide concentration

Hydrogen peroxide was measured via a horseradish peroxidase (HRPO)-mediated oxidation of phenol red, leading to the formation of a compound measurable at 610 nm. Slices of fresh tissue from the LV were incubated for 30 min at 37°C in phosphate buffer at a concentration of 10 mmol/L (NaCl 140 mmol/L and dextrose 5 mmol/L). The supernatants were transferred to tubes containing 0.28 mmol/L phenol red and 8.5 U/mL HRPO. After incubating for 5 min, 1 mol/L NaOH was added, and the absorbance values of the solution were measured. The results were expressed in nanomoles H_2O_2/g tissue.[19]

Protein concentration determination

The protein concentration was measured by the method of Lowry *et al.*[20] using bovine serum albumin (BSA) as the standard.

Statistical analysis

The data were compared expressed in mean ± standard error of the mean (SEM). The significant differences among measured variables were assessed by two-way analysis of variance (ANOVA), followed by post hoc Tukey test using the SIGMA PLOT software (Systat Software, Inc., Illinois, Chicago, USA). The correlation between two variables was analyzed by Pearson's correlation. All P-values reported are two-tailed and $P < 0.05$ was considered significant.

RESULTS

Morphometric parameters

Table 1 summarizes the morphometric characteristics of the animals on the day they were killed. Final body weight was lower ($P < 0.001$) in the MCT groups than the controls, starting at day 7. Right ventricle weight to body weight ratio (RVW/BW) of MCT 21 day and MCT 31 day groups was significantly higher than their respective controls ($P < 0.01$), whereas left ventricle plus interventricular septum weight to body weight ratio (LVW/BW) was unaffected by MCT treatment. The degree of cardiac hypertrophy was determined as the heart weight to body weight ratio (HW/BW), this parameter showed an increase ($P < 0.05$) in MCT 21 day rats (by 14%), as well as in MCT 31 day rats (by 59%, $P < 0.01$) (Table 1). Lung congestion was significantly higher ($P < 0.01$) in

Table 1 Morphometric parameters evaluated 7, 21 and 31 days after monocrotaline (MCT) or saline (control) injections

Time	Group (*n*)	BW	RVW/BW	LVW/BW	HW/BW	LC
Day 7	Control (8)	227.5 ± 12.2	0.68 ± 0.03	2.4 ± 0.05	3.08 ± 0.19	5.4 ± 0.65
	MCT (8)	182.5 ± 14.1***	0.63 ± 0.04	2.4 ± 0.05	3.03 ± 0.15	7.09 ± 0.87
Day 21	Control (6)	280.8 ± 40.8	0.45 ± 0.02	2.2 ± 0.11	2.65 ± 0.09	5.09 ± 1.08
	MCT (8)	240.6 ± 41.4***	0.83 ± 0.04**	2.2 ± 0.16	3.03 ± 0.29*	9.05 ± 1.89**
Day 31	Control (8)	305.0 ± 24.5	0.55 ± 0.02	2.1 ± 0.07	2.65 ± 0.09	5.87 ± 1.34
	MCT (6)	195.0 ± 39.7***	2,01 ± 0.20**	2.2 ± 0.41	4.21 ± 0.50**	11.21 ± 4.62**

Values are means ± standard error of the mean (SEM). *Control compared with MCT, $P < 0.05$; **Control compared to MCT, $P < 0.01$; ***Control compared with MCT, $P < 0.001$. Two-way analysis of variance with post hoc Tukey test. BW, body weight (g); HW, heart weight (mg); LC, lung congestion; LVW, left ventricular weight (mg); RVW, right ventricular weight (mg).

MCT 21 day and MCT 31 day rats than in their respective controls (Table 1). RV hypertrophy positively correlated with lung congestion ($r = 0.68$, $P < 0.01$).

Echocardiographic analysis

Pulmonary vascular resistance

Representative echocardiograms of pulmonary artery flow can be seen for the control groups at 7, 21, and 31 days (Fig. 1a–c) and also in the respective MCT groups (Fig. 1d–f). Acceleration-to-ejection time ratio (AT/ET) through the pulmonary artery was reduced ($P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively) in all MCT groups (7 days, 21 days and 31 days, respectively) when compared with their respective controls (Fig. 2). AT/ET ratios negatively correlated with RV hypertrophy ($r = –0.76$, $P < 0.01$).

Left ventricle function

The analyzed parameters were the myocardial performance index and diastolic and systolic functions. The only changes in the analyzed parameters were in the MCT 31 day group.

Myocardial performance index

An important increase in this parameter was shown in the MCT 31 day group in relation to its control ($P < 0.001$) (Table 2). A strong positive correlation was found between MPI and RV hypertrophy ($r = 0.75$, $P < 0.001$).

Diastolic function

Flow velocity of the E and A waves through the mitral valve was significantly lower ($P < 0.001$ and $P < 0.01$, respectively) in the MCT 31 day group than in respective controls (Table 2). Representative echocardiograms of the mitral flow can be seen for the control groups at 7, 21, and 31 days (Fig. 1g–i), and the same can be seen for the monocrotaline groups (Fig. 1j–l).

Systolic function

Systolic and diastolic diameters of the LV showed a reduction ($P < 0.001$) in the MCT 31 day group in relation to their respective control group. The LV posterior wall thickness was found to be enhanced during diastole ($P < 0.05$), and this did not change in systole. The LV fractional area change (FAC) as well as fractional shortening (LVFS) were augmented ($P < 0.001$ for both) in the MCT 31 day group as compared with their respective controls.

Oxidative stress measurements

Left ventricle hydrogen peroxide concentration showed an increase ($P < 0.05$) in the MCT 21 day and 31 day groups with respect to their control groups (Fig. 3a), and the oxidized-to-total glutathione ratio did not differ among groups (Fig. 3b). Ascorbic acid concentration in the LV was reduced ($P < 0.05$) in the MCT 31 day group when compared to its respective control (Table 3). Thioredoxin reductase activity (TrxR) in the LV was highest in the MCT 31 day group as compared with the other groups.

DISCUSSION

The main finding of our study was the progressive increase of pulmonary vascular resistance throughout the experimental protocol, triggering right ventricle hypertrophy and culminating in systolic and diastolic left ventricular dysfunction at 31 days after MCT administration. At this time point, an increased effort of the myocardium associated with adaptations of the intracellular antioxidant system in the left ventricle was seen.

The experimental model applied in the present study has been largely used for the development of pulmonary hypertension, followed by pressure overload to the right ventricle, hypertrophy and eventually congestive heart failure in rats.[21,22] Studies conducted by our group showed that cardiac hypertrophy after 21 days of the MCT injection were associated with changes in the activity of antioxidant enzymes in the right ventricle and lung.[6,23] Thus, we proposed to evaluate the changes generated by the development of pulmonary hypertension at distinct moments (7, 21 and 31 days) to follow the evolution of the RV hypertrophy and failure. These three moments were chosen based on studies, in this experimental model, that do not show cardiac hypertrophy after 7 days,[11] exhibit hypertrophic adaptation[6] at 21 days and show signs of heart failure at 28 days.[24] In our study, these data were corroborated since there was RV hypertrophy from the 21st day after MCT injection. An indicator of an increase in pulmonary vascular resistance (PVR) is the ratio of the acceleration time (AT) to the ejection time (ET). A strong negative correlation was observed between the AT/ET ratio and right ventricular hypertrophy, and a positive correlation with lung congestion was observed, strengthening the association between increased PVR, RV hypertrophy and heart failure.

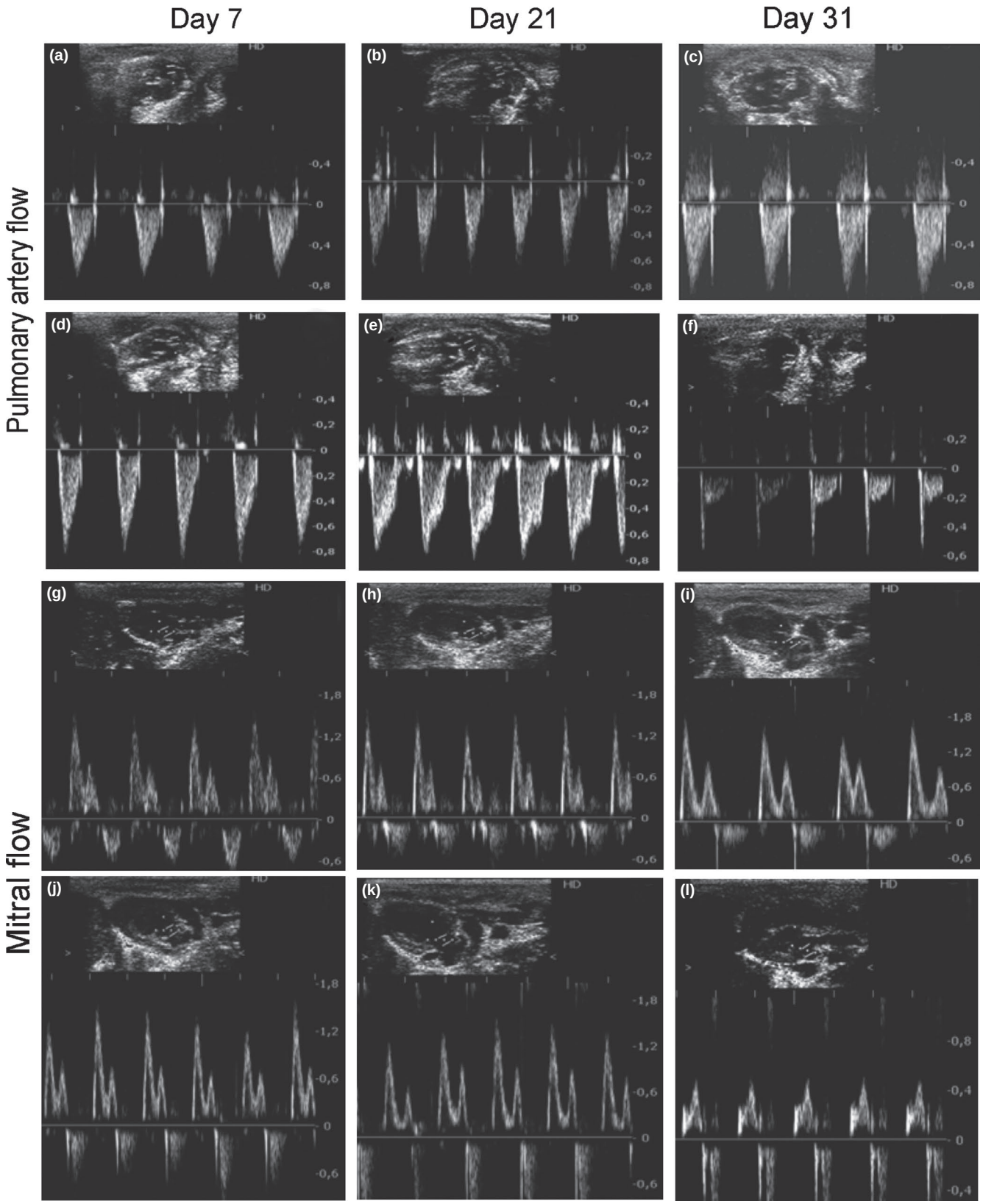

Fig. 1 Representative echocardiograms of pulmonary artery flow (in cm/s) in control 7, 21, 31 days (a–c) and monocrotaline groups (d–f); mitral flow (in cm/s), peak E and A waves, in control groups 7, 21, 31 days (g–i) and monocrotaline groups (j–l).

The AT/ET ratio was reduced in the MCT groups (Fig. 2) from the first week of treatment. This change of flow was also observed by Kato *et al.*[24] in the same model 28 days after MCT administration. These findings indicate increased resistance in pulmonary circulation.[25] As a result of the increased PVR, the blood flow through the pulmonary artery demonstrated an evident change in its classic pattern (Fig. 1a–f) in the MCT groups from day 21, being more marked at the end of the protocol (day 31). The wave pattern found in this study is characterized as late systolic notch[26] and differs from a study conducted by Jones,[27] which showed a characteristic pattern of midsystolic notch 42 days after MCT administration. We attribute this difference in results to the fact that this model is time-dependent, and midsystolic notch denotes a higher degree of PVR.[28]

Although the MCT model is known to induce right ventricular dysfunction in a time- and dose-dependent fashion, significant changes were observed in left ventricle systolic and diastolic function. The performance myocardial index (PMI) has been used as a parameter of cardiac effort in both clinical studies and in experimental animals.[29,30] A significant increase was found in the PMI (132%, $P < 0.001$) in the MCT 31 day group compared to the control 31 day group. A strong positive correlation between RV hypertrophy and the PMI ($r = 0.75$, $P < 0.001$) was observed, suggesting that

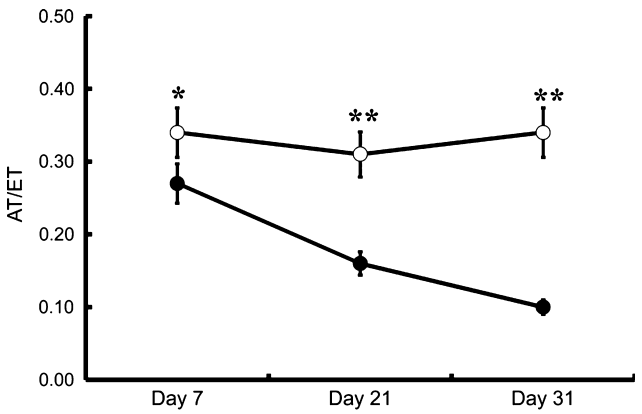

Fig. 2 Acceleration-to-ejection time ratio (AT/ET) through the pulmonary artery. *$P < 0.05$ and **$P < 0.01$ compared with the respective control groups. Two-way analysis of variance with post hoc Tukey test. —o—, control; —●—, monocrotaline. The data are expressed as mean ± standard error of the mean (SEM).

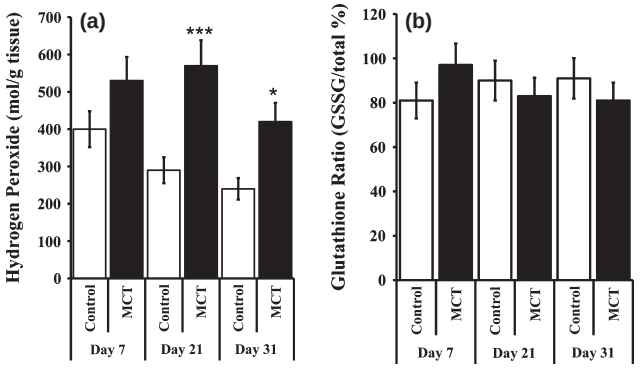

Fig. 3 The data are expressed as means ± standard error of the mean (SEM). Hydrogen peroxide concentration in the left ventricle (a); reduced-to-total glutathione ratio in the left ventricle (b).*$P < 0.05$ compared to the controls and **$P < 0.01$ compared with the control, respectively. Two-way analysis of variance with post hoc Tukey test.

Table 3 Antioxidants results in left ventricle of the distinct groups

	Ascorbic acid (μmol/L)	Tioredoxin red. (nmol/mg prot)
Day 7		
Control	23.92 ± 6.02 (5)	0.11 ± 0.05 (5)
MCT	35.27 ± 5.10 (5)	0.10 ± 0.04 (6)
Day 21		
Control	21.75 ± 5.54 (4)	0.13 ± 0.03 (6)
MCT	25.69 ± 4.60 (4)	0.13 ± 0.02 (6)
Day 31		
Control	29.47 ± 4.88 (5)	0.14 ± 0.04 (6)
MCT	9.58 ± 3.17* (4)	0.21 ± 0.05* (6)

Values are means ± standard error of the mean (SEM) (*n*). *Compared with all groups, $P < 0.05$. MCT, monocrotaline. Two-way analysis of variance with post hoc Tukey test.

Table 2 Echocardiography parameters evaluated 7, 21 and 31 days after monocrotaline (MCT) or saline (control) injections

	Day 7		Day 21		Day 31	
	Control	MCT	Control	MCT	Control	MCT
MPI	0.25 ± 0.03	0.32 ± 0.05	0.30 ± 0.03	0.33 ± 0.04	0.27 ± 0.03	0.65 ± 0.01***
Peak E	1.50 ± 0.03	1.40 ± 0.04	1.50 ± 0.07	1.40 ± 0.03	1.51 ± 0,07	0.80 ± 0.09***
Peak A	0.80 ± 0.04	0.70 ± 0.05	0.80 ± 0.04	0.80 ± 0.03	0.98 ± 0.05	0.60 ± 0.07**
LVSD	0.32 ± 0.01	0.35 ± 0.02	0.30 ± 0.02	0.28 ± 0.01	0.33 ± 0.01	0.21 ± 0.02***
LVDD	0.70 ± 0.01	0.70 ± 0.01	0.60 ± 0.02	0.60 ± 0.01	0.73 ± 0.01	0.50 ± 0.02***
EDPWT	0.13 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.14 ± 0.01*
ESPWP	0.22 ± 0.01	0.21 ± 0.01	0.23 ± 0.01	0.21 ± 0.01	0.24 ± 0.01	0.25 ± 0.01
FAC	0.70 ± 0.02	0.70 ± 0.02	0.80 ± 0.01	0.80 ± 0.02	0.52 ± 0.03	0.80 ± 0.07***
LVFS	54.00 ± 1.44	49.00 ± 2.48	56.00 ± 1.84	57.00 ± 1.86	53.00 ± 1.77	68.00 ± 5.13***

Values are means ± standard error of the mean (SEM). *Control compared with MCT, $P < 0.05$; **Control compared with MCT, $P < 0.01$; ***Control compared with MCT, $P < 0.001$. Two-way analysis of variance with post hoc Tukey test. EDPWP, End diastolic posterior wall thickness (cm); ESPWP, End systolic posterior wall thickness (cm); FAC, Fractional area change; LVDD, Left ventricle diastolic diameter (cm); LVFS, Left Ventricle Fraction Shortening (%); LVSD, Left ventricle systolic diameter (cm); MPI, Myocardial performance index.

hypertrophy is related to the increase in resistance promoted by PAH and consequent increase of the RV workload,[31] influencing the LV mechanics.

Bogaard *et al.*[32] relate the increase in the RV afterload, due to pulmonary hypertension, to a possible reversal of the interventricular septum toward the LV, resulting in a reduction of the left ventricular chamber. Our results related to systolic function present a reduction of the LV end systolic and diastolic diameters; however, the increase of the shortening fraction (LVFS) and consequent increase in the fractional area change (FAC) (Table 2) suggest an adaptation in the attempt to preserve the stroke volume.

In the analysis of diastolic function, the values of the peak E and A waves presented differences (Table 2). The E and A waves are dependent on venous return caused by the relation between RV stroke volume and pulmonary resistance and the capacity of the left atrium to maintain its pump function, respectively. By correlating the index of hypertrophy of the RV with the E wave, a significant difference was found ($P < 0.05$) with a strong negative correlation ($r = -0.75$). These data suggest that the higher the RV hypertrophy, the lesser blood flows through the mitral valve during rapid filling. These data are suggestive that both systolic and diastolic LV functions are being determined by the degree of severity of the PVR.

The process of RV hypertrophy and failure has been largely related to ROS and oxidative stress.[7,11,23] A study conducted by Sam *et al.*[33] demonstrated an increase of ROS production associated with changes in the activity of antioxidant enzymes in the myocardium of patients with nonischaemic dilated cardiomyopathy. Previously, Pichardo *et al.*[7] showed an increase in lipid peroxidation and reduction of non-enzymatic antioxidant defences such as α-tocopherol, but with no change of the enzymatic defences in the hypertrophied RV. Our study shows a clear increase in the concentration of hydrogen peroxide in the LV at 21 and 31 days after administration of MCT, at which point major changes are seen in RV function. Studies conducted by our group using other experimental models show hydrogen peroxide as a ROS having a flag potential for adjustments related to hypertrophy and/or cell death depending on its intracellular concentration.[34,35]

There are many antioxidants that may influence the hypertrophic adaptive response by means of their ability of controlling ROS concentrations. Ahsan[36] describes thioredoxin (TRX) and glutaredoxin (GRX) as the two main antioxidant systems involved in redox regulation to protect cells from oxidative stress by being involved in the reduction process of hydrogen peroxide to water. A significant increase was observed ($P < 0.05$) in the activity of thioredoxin reductase (TrxR) in the MCT 31 day group in relation to its control. TrxR is an important enzyme that has been related to cardiac dysfunction[37] and has an important role in the regeneration of TRX. In this study, the increased activity of TrxR at 31 days may result from the sustained high concentrations of hydrogen peroxide in the LV as well the reduction in ascorbic acid in this time. Ascorbic acid acts as an intracellular hydrosoluble non-enzymatic antioxidant by its ability to donate electrons[38] and thus contributes to the maintenance of the concentration of NADPH required for the regeneration of GSH. The decrease of ascorbic acid observed in this study may represent the consumption of this important defence for the regeneration of reduced glutathione. In these time points, the redox balance (GSSG/GSH) is kept in equilibrium, probably at the expense of ascorbic acid and possibly other antioxidants recruited to cellular defence. It is possible that at 21 days other antioxidant defences that were not evaluated may be involved in the reduction of hydrogen peroxide.

Our findings showed that LV dysfunction followed by RV changes due pulmonary hypertension at 31 days after injection of monocrotaline is associated with hydrogen peroxide increase and a sharp reduction in vitamin C in LV. These data support the design of further studies to understand the mechanisms involved. Therapies relieving oxidative stress in the LV in conjunction with PAH treatment might be relevant.

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