Comparison of two experimental models of pulmonary hypertension*

Comparação de dois modelos experimentais de hipertensão pulmonar

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Abstract

Objective: To compare two models of pulmonary hypertension (monocrotaline and monocrotaline+pneumonectomy) regarding hemodynamic severity, structure of pulmonary arteries, inflammatory markers (IL-1 and PDGF), and 45-day survival. **Methods:** We used 80 Sprague-Dawley rats in two study protocols: structural analysis; and survival analysis. The rats were divided into four groups: control; monocrotaline (M), pneumonectomy (P), and monocrotaline+pneumonectomy (M+P). In the structural analysis protocol, 40 rats (10/group) were catheterized for the determination of hemodynamic variables, followed by euthanasia for the removal of heart and lung tissue. The right ventricle (RV) was dissected from the interventricular septum (IS), and the ratio between RV weight and the weight of the left ventricle (LV) plus IS (RV/LV+IS) was taken as the index of RV hypertrophy. In lung tissues, we performed histological analyses, as well as using ELISA to determine IL-1 and PDGF levels. In the survival protocol, 40 animals (10/group) were followed for 45 days. **Results:** The M and M+P rats developed pulmonary hypertension, whereas the control and P rats did not. The RV/LV+IS ratio was significantly higher in M+P rats than in M rats, as well as being significantly higher in M and M+P rats than in control and P rats. There were no significant differences between the M and M+P rats regarding the area of the medial layer of the pulmonary arteries; IL-1 and PDGF levels; or survival. **Conclusions:** On the basis of our results, we cannot conclude that the monocrotaline+pneumonectomy model is superior to the monocrotaline model.

Keywords: Monocrotaline; Hypertension, pulmonary; Pneumonectomy; Interleukin-1; Receptor, platelet-derived growth factor beta.

Resumo

Objetivo: Comparar dois modelos de hipertensão pulmonar (monocrotalina e monocrotalina+pneumonectomia) em relação à gravidade hemodinâmica, estrutura de artérias pulmonares, marcadores inflamatórios (IL-1 e PDGF) e sobrevida em 45 dias. **Métodos:** Foram utilizados 80 ratos Sprague-Dawley em dois protocolos de estudo: análise estrutural e de sobrevida. Os animais foram divididos em quatro grupos: controle, monocrotalina (M), pneumonectomia (P) e monocrotalina+pneumonectomia (M+P). Para a análise estrutural, 40 animais (10/grupo) foram cateterizados após 28 dias para a medição dos valores hemodinâmicos e sacrificados, obtendo-se tecidos cardíaco e pulmonar. O ventrículo direito (VD) foi dissecado do septo interventricular (SI), e a relação do peso do VD e do peso do ventrículo esquerdo (VE) com o SI foi obtida como índice de hipertrofia de VD. No tecido pulmonar, foram realizadas análises histológicas e dosados IL-1 e PDGF por ELISA. Para o estudo de sobrevida, 40 animais (10/grupo) foram observados por 45 dias. **Resultados:** Os grupos M e M+P apresentaram hipertensão pulmonar em relação aos demais. Houve um aumento significativo da relação VD/VE+S no grupo M+P em relação aos demais. Não houve diferenças significativas entre os grupos M e M+P quanto à área da camada média das artérias pulmonares, dosagens de IL-1 e PDGF ou sobrevida. **Conclusões:** Baseados nos resultados, não podemos afirmar que o modelo de monocrotalina+pneumonectomia é superior ao modelo de monocrotalina.

Descritores: Monocrotalina; Hipertensão pulmonar; Pneumonectomia; Interleucina-1; Receptor beta de fator de crescimento derivado de plaquetas.

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Introduction

Pulmonary arterial hypertension (PAH) comprises a group of diseases that share pathological similarities but differ in pathophysiology and prognosis.[1] Clinically, PAH is characterized by symptoms of dyspnea, chest pain, and syncope, causing progressive limitation, right heart failure, and death.[2,3] Idiopathic PAH is rare, has a peak incidence in the fourth decade of life, and mainly affects females; however, other forms of PAH, such as schistosomiasis-associated PAH, are potentially more prevalent and can become a public health problem.[1,4,5]

According to the 4th World Symposium on Pulmonary Hypertension,[6] held in Dana Point, CA, USA, PAH is defined by right heart catheterization measurements. Therefore, in a patient with PAH, cardiac catheterization should show a mean pulmonary artery pressure (mPAP) ≥ 25 mmHg and a pulmonary artery occlusion pressure ≥ 15 mmHg.

The disease is the result of complex changes that lead to structural modifications in pulmonary arteries and arterioles. These changes ultimately cause the clinical manifestations of PAH. The progression of vascular changes usually occurs after one or more offending stimuli in a susceptible individual, and, despite the drugs available for treatment, PAH remains a fatal disease.[7,8]

Experimental models of PAH have allowed the development of all of the therapeutic alternatives that are currently available, and the monocrotaline model remains the most widely used.[9-11] The use of seeds of *Crotalaria spectabilis*, the plant from which monocrotaline is derived, was described more than 40 years ago. Initially, the animals were fed the seeds, subsequently developing PAH.[12] Currently, monocrotaline is administered subcutaneously; after undergoing oxidation in the liver, monocrotaline produces its pyrrole metabolite and reaches the lung, where it causes lesions in the pulmonary circulation, the targets being various proteins and peptides of the vascular endothelium.[13]

Four hours after the substance is administered, it is possible to see a relative increase in the media of intra-acinar pulmonary arteries, which is due to muscle contraction. Within 8-16 h after administration, mononuclear inflammatory infiltrates are seen in the adventitia of arteries and veins, resulting in vasculitis. After approximately 22 days, there is right ventricular hypertrophy and marked mononuclear vasculitis in intra-acinar arteries and veins.[14] Therefore, after monocrotaline causes an intense inflammatory reaction in the pulmonary arteries and arterioles, the process of vascular remodeling, mainly associated with medial thickening, begins.[15,16] These findings have led various authors to study the effect of monocrotaline on the rat pulmonary circulation and its association with different interventions.[9-11,17-20]

Because of its simplicity, the monocrotaline model is widely used for testing new drugs; however, the list of drugs that can reverse monocrotaline-induced PAH is extensive, paradoxically including drugs that can cause PAH in humans.[21] Therefore, the problem with this model is that it does not reliably represent all of the changes that occur in human PAH, especially because there is no significant endothelial proliferation.[7]

Studies conducted more recently have addressed the hypothesis that the use of a systemic-pulmonary shunt or pneumonectomy—producing an increase in pulmonary blood flow—in combination with the administration of monocrotaline can lead to exacerbation of the resulting findings in the pulmonary circulation.[22-24] Although such studies have suggested that the model combining monocrotaline and increased blood flow is superior, no direct comparisons have been made in order to set the standard for use in future studies. Therefore, the objective of the present study was to compare the monocrotaline model with the model combining monocrotaline and increased blood flow (following left pneumonectomy) in adult rats, in terms of hemodynamic severity, structural changes in the pulmonary arteries, inflammatory markers, and 45-day survival.

Methods

All animals were handled humanely, in accordance with international standards for animal care.[25] The study was approved by the Research Ethics Committee of the University of São Paulo School of Medicine, located in the city of São Paulo, Brazil.

Two study protocols were performed: one for structural analysis and one for survival analysis. Each protocol included 40 Sprague-Dawley rats (weight, 250-300 g) divided into four groups: the control group, in which the animals were given a subcutaneous injection of saline (1 mL/kg) at the study outset (D0); the monocrotaline (M) group,

in which the animals were given a subcutaneous injection of monocrotaline (Sigma-Aldrich, St. Louis, MO, USA; 60 mg/kg) on D0; the left pneumonectomy (P) group, in which the animals underwent left pneumonectomy 7 days prior to D0 and received a subcutaneous injection of saline (1 mL/kg) on D0; and the monocrotaline+left pneumonectomy (M+P) group, in which the animals underwent left pneumonectomy 7 days prior to D0 and received a subcutaneous injection of monocrotaline (60 mg/kg) on D0.

The pneumonectomy technique used in the present study was as follows:

- The animals were anesthetized with 2.5% isoflurane in an anesthesia chamber, anesthesia being maintained throughout the surgical procedure.
- The animals were intubated with a 14-gauge Jelco® catheter (Johnson & Johnson, São José dos Campos, Brazil) and coupled to a Harvard rodent ventilator (model 683; Harvard Apparatus Co., South Natick, MA, USA), RR being maintained at 80 breaths/min and tidal volume being maintained at 10 mL/kg of body weight.
- The animals were placed in the left lateral decubitus position, the left infra-axillary region was shaved, and, subsequently, a 3-cm incision was made between the fourth and fifth intercostal spaces, running parallel to the spaces.
- The skin, subcutaneous cellular tissue, and intercostal muscle planes were sectioned until the thoracic cavity was reached, at which point the retractor was placed between the ribs.
- The left lung was freed from its ligaments and subsequently drawn out of the cavity.
- After en bloc ligation of the hilum with 2-0 cotton sutures, the left lung was sectioned.
- The muscle planes were closed with continuous 3-0 nylon sutures (Ethicon, São Paulo, Brazil), and the skin was sutured with the same suture material.

The analyses were performed 28 days after the injection of monocrotaline or saline. After deep sedation with xylazine hydrochloride (0.3 mg/kg, i.p.; Rompun®; Bayer, Leverkusen, Germany) and ketamine hydrochloride (10 mg/kg, i.p.; Ketalar®; Pfizer, New York, NY, USA), the animals were weighed. Hemodynamic measurements were performed, being followed by euthanasia (abdominal aortic bleeding) and removal of heart and lung tissue.

The hemodynamic measurements were performed by inserting an umbilical catheter into the external jugular vein, the catheter being connected to a pressure transducer (HP 1295C; Hewlett-Packard, Palo Alto, CA, USA) coupled to a hemodynamic monitor (Monitox Dx 2020; Hewlett-Packard), in accordance with a previously described technique.[26] The mPAP was thus measured.

The right ventricle (RV) was dissected from the left ventricle (LV), the interventricular septum (IS) having remained attached to the LV. The ratio between RV weight and LV+IS weight was taken as the index of right ventricular hypertrophy.[19]

To investigate the degree of inflammation, we determined IL-1 and PDGF levels. To measure peptide levels, we used a capture ELISA with a commercial kit including anti-mouse IL-1 and PDGF (R&D System Inc., Minneapolis, MN, USA).[27] Peptide levels were measured in frozen lung samples.

We employed 96-well plates (Costar; Corning Inc., Cambridge, MA, USA), which were sensitized with 100 µL of monoclonal antibody and incubated for 18 h at 4°C. Subsequently, in order to prevent nonspecific bindings, the plates were blocked with 300 µL of 2% BSA and incubated for 2 h at 37°C. After blocking, sample and standards diluted in PBS were added at 100 µL/well. Two wells were filled only with PBS for use as a blank. The plates were incubated for 18 h at 4°C.

After incubation, we added 100 µL of biotinylated conjugated antibody at a pre-established concentration, and the plates were incubated for 3 h at 37°C. Subsequently, we added 100 µL of streptavidin-HRP (1:250; R&D Systems, Minneapolis, MN, USA) to each well, and the plates were incubated for 30 min at 37°C. At each step, the plates were washed six times in wash buffer (PBS and Tween 20).

For color development, we added 100 µL of developer (hydrogen peroxide and tetramethylbenzidine) to each well, and the plates were incubated for 5 or 60 min, depending on the cytokine, at 37°C. The reaction was stopped by the addition of 50 µL of 30% sulfuric acid to each well, followed by gentle agitation of the plates. The plates were read by an ELISA reader (Power Wave; Bio-Tek Instruments Inc., Winooski, VT, USA) with a 450-nm filter.

For histological quantification, five different randomly selected fields were examined (magnification, ×400) after the tissue had been stained with Miller's stain, which allows the visualization of elastic fibers. For this quantification, we used an image analysis system (Carl Zeiss; MicroImaging GmbH, Göttingen, Germany) and AxioVision 40 version 4.7.1.0 software, 2006-2008 (Carl Zeiss Imaging Solutions GmbH, Jena, Germany), which allows quantitative geometric and quantitative densitometric measurements. Specific thresholds were established for each slide. The histological findings are expressed as area (μm^2).[28]

The objective of the survival protocol, with 40 rats also divided into control, P, M, and M+P rats, was to investigate differences in survival among the models. The animals were followed for 45 days, and the date of death of each rat was recorded. All of the remaining animals were sacrificed on day 45.

For the statistical analysis, ANOVA with post hoc Bonferroni correction was used in order to compare continuous variables among the groups. Proportional variables were analyzed by the chi-square test or Fisher's exact test, as appropriate. Values of $p < 0.05$ were considered significant.

To estimate survival over time, we used the Kaplan-Meier method, the survival curves being compared by the log-rank test.

Results

Invasive hemodynamic measurements revealed that M and M+P rats developed PAH, whereas control and P rats did not (Figure 1).

As can be seen in Figure 2A, the RV weight was significantly higher in the rats that developed PAH (i.e., M and M+P rats) than in those that did not (i.e., control and P rats), as well as being significantly higher in M+P rats than in M rats. There were no differences among the groups regarding LV+IS weight (Figure 2B); this caused the index of RV hypertrophy (RV/LV+IS ratio) to be significantly higher in M+P rats than in those in the remaining groups (Figure 2C), similarly to the distribution of RV weight.

The medial area was significantly larger in M and M+P rats than that in control rats ($p = 0.013$; Figure 2D). There was no intimal proliferation in M or M+P rats (Figure 3).

The IL-1 levels were significantly higher in P, M, and M+P rats than in control rats (Figure 4A). Although the PDGF levels were also different among the groups ($p = 0.049$), our post hoc analysis did not allow us to determine the significance of the differences (as it did for the IL-1 levels); nevertheless, the distribution of the data suggests that the patterns are the same (Figure 4B).

Although survival was significantly lower in M and M+P rats than in control and P rats ($p < 0.05$), there were no differences between M and M+P rats (Figure 5).

Discussion

Our study showed that both the monocrotaline model and the model combining monocrotaline and increased blood flow produced by pneumonectomy induce PAH in rats. Analogously and consequently, we observed that RV weight and the index of RV hypertrophy were higher in M+P rats than in M rats, as well as being higher in M and M+P rats than in control and P rats.

It is of note, however, that the index of RV hypertrophy (RV/LV+IS ratio) does not represent PAH in an absolute sense, because monocrotaline can also have a direct effect on the heart.[21] Nevertheless, the finding of similar LV weights in the different groups does not suggest the presence of monocrotaline-induced cardiac abnormalities in our study.

The M and M+P rats showed no differences in medial area when compared with each other.

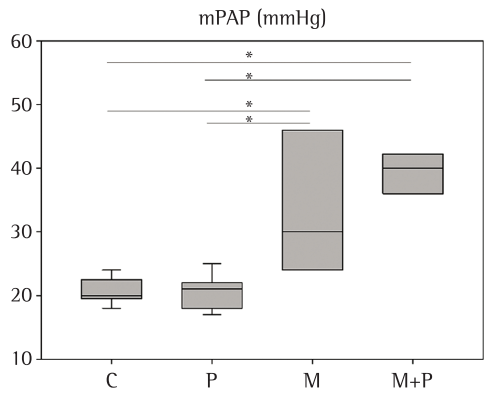

Figure 1 - Comparison of control (C), monocrotaline (M), pneumonectomy (P), and monocrotaline+pneumonectomy (M+P) rats regarding mean pulmonary artery pressure (mPAP) in mmHg. *$p < 0.001$.

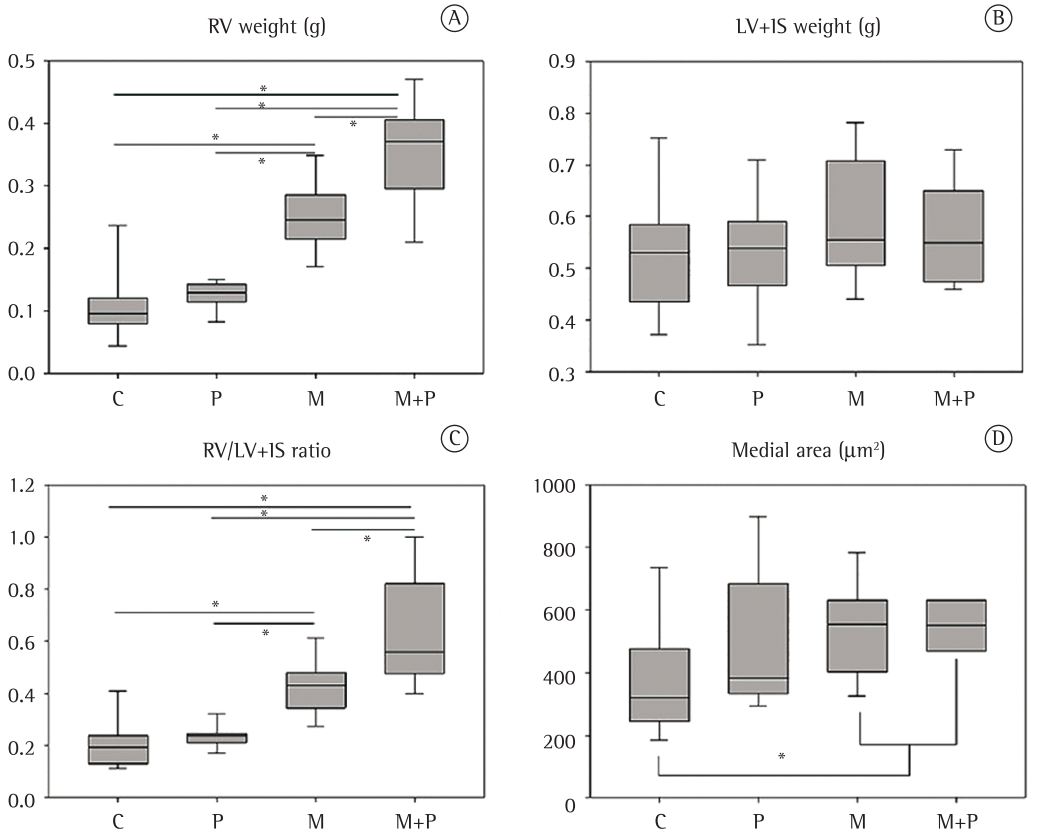

Figure 2 - Comparison of control (C), monocrotaline (M), pneumonectomy (P), and monocrotaline+pneumonectomy (M+P) rats. In A, right ventricle (RV) weight in g, *p < 0.001; in B, left ventricle plus interventricular septum (LV+IS) weight in g; in C, the RV/LV+IS ratio, *p < 0.001; in D, medial area in µm², *p = 0.013.

However, when compared with control rats, M and M+P rats showed significantly larger areas (p = 0.013), which confirmed that the two models produce pathological changes typically caused by PAH. Control and P rats showed no significant differences in medial area. One important finding is that there was no intimal proliferation in any of the groups, which is at odds with the findings of other studies.[23,29]

Regarding inflammatory markers, IL-1 levels were significantly higher in P, M+P, and M rats than in control rats, and a similar pattern was found for PDGF levels. This demonstrates that surgical manipulation alone increases the levels of these inflammatory markers, which limits their use in the comparison of the models, although it has been demonstrated that these markers are increased in individuals with PAH.[17,30] However, it is of note that, in our study, the levels of these markers were measured in whole lung samples, and no microdissection technique was used in order to analyze isolated vascular material.[15] This means that any inflammatory phenomenon occurring in the lungs is reflected in the methodology employed, a factor that constitutes a limitation of the methodology.

The experimental models of PAH studied do not mimic human PAH closely enough. This is due to several factors, among which is the speed of disease onset, which occurs over years in humans but in weeks in rats. It is therefore logical to think that the pathophysiological mechanisms are different.[21] Consequently, other models, such as the model combining monocrotaline injection and increased pulmonary blood flow produced by systemic-pulmonary shunts or pneumonectomy, have been studied.[22-24]

In 1996, Tanaka et al.[22] tested the hypothesis that, by raising pulmonary artery blood pressures to systemic levels, the effects of monocrotaline on pulmonary circulation could be increased. The authors found that the creation of a subclavian-

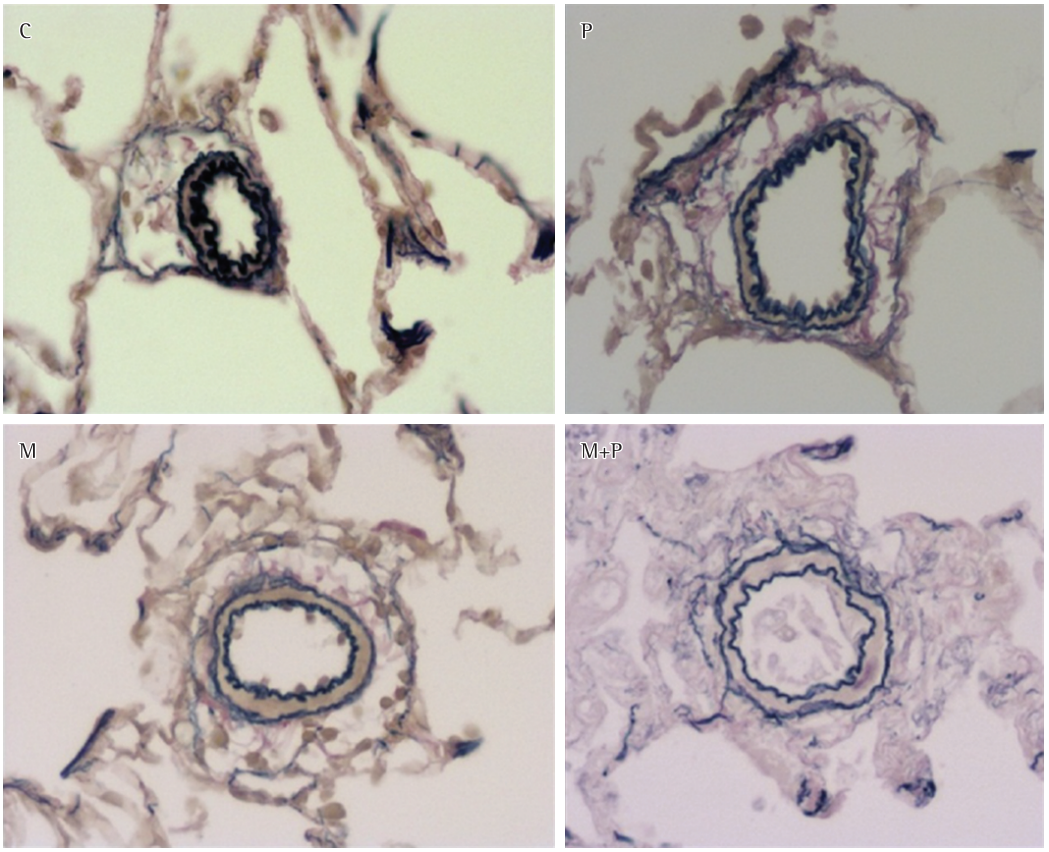

Figure 3 - Sample images of histological sections of the media of acinar arterioles (magnification, ×400). C: control group; M: monocrotaline group; P: pneumonectomy group; and M+P: monocrotaline+pneumonectomy group.

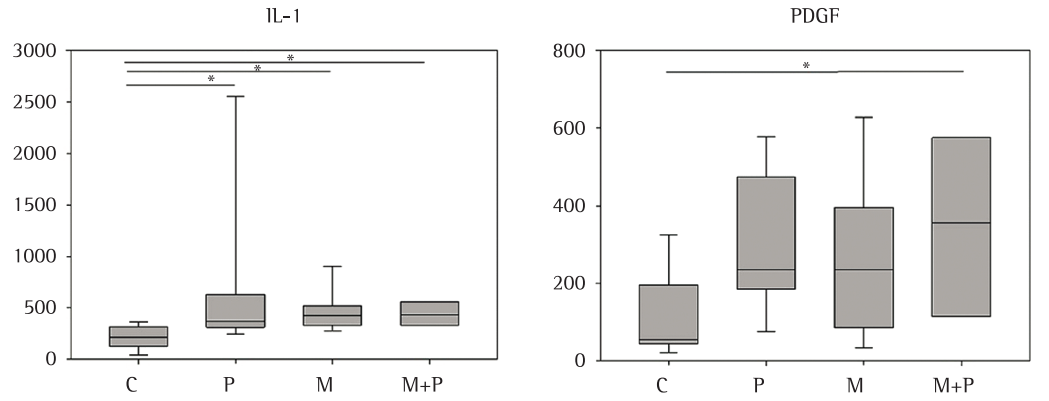

Figure 4 - In A, IL-1 levels in pg/mL, *p < 0.05; in B, PDGF levels in pg/mL, *p = 0.049. C: control group; M: monocrotaline group; P: pneumonectomy group; and M+P: monocrotaline+pneumonectomy group.

pulmonary artery anastomosis in rats, with subsequent injection of monocrotaline, led to intimal lesions in large pulmonary arteries. The authors demonstrated that, in the absence of endothelial injury, there was no vascular remodeling (at least not in large blood vessels), even when the animals were submitted to systemic pressures.

In a subsequent study,[23] left pneumonectomy was followed by injection of monocrotaline. The authors of that study found intimal lesions in distal pulmonary arteries, a finding that was

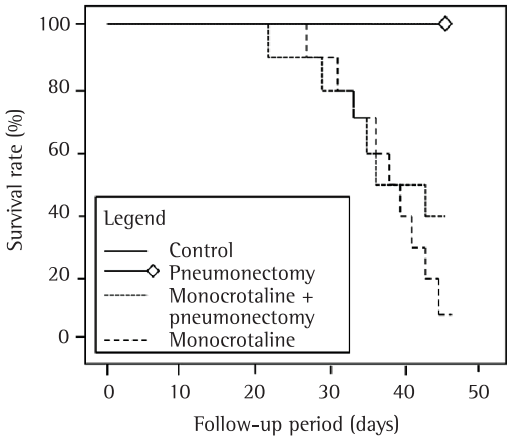

Figure 5 - Kaplan-Meier survival curve. There were no significant differences between monocrotaline and monocrotaline+pneumonectomy rats.

attributed to increased blood flow in the arteries of the remaining lung. The authors hypothesized that shear stress was responsible for the changes observed. The two abovementioned studies, as well as those involving other animal species, showed that, in the absence of endothelial injury, increased blood flow following pneumonectomy does not cause significant increases in pulmonary artery pressure levels.[24] In humans, histological examinations performed 1-5 years after pneumonectomy showed only medial hypertrophy, without intimal lesions in the pulmonary arteries.[24] Therefore, the histological changes caused by these experimental models are potentially similar to the typical findings in human PAH, especially because of increased neointimal proliferation.[18-20,22]

White et al.[29] compared the monocrotaline+left pneumonectomy model of pulmonary hypertension in younger rats with the pathological lesions observed in autopsy studies of human PAH. The authors hypothesized that, in younger rats, the changes observed by Tanaka et al.[22] would be even more pronounced. They found complex, proliferative perivascular lesions in the rats studied.

The rats used in our study were not as young as those used by White et al.,[29] who observed complex, proliferative perivascular lesions in their rats. Tanaka et al., who pioneered this model,[22] found intimal lesions. However, their findings were different from those reported by White et al. The difference was likely due to the younger age of the rats used, given that the methods employed in the two studies were the same. In older rats, postpneumonectomy lung growth is less pronounced, as are the inflammatory effects of monocrotaline. Therefore, in the study by White et al., the observed phenomena might be due to compensatory lung growth following pneumonectomy.[22,29]

Although we found no significant differences between M+P and M rats regarding hemodynamic changes or medial thickness, we found that RV hypertrophy was greater in M+P rats (Figure 2). This finding alone does not allow us to conclude that the monocrotaline+pneumonectomy model is more severe than is the monocrotaline model, because survival was the same. No author had tested the difference in survival among the various models. We believe that this is the parameter that should be used for determining the actual severity of the disease produced by experimental models.

We can conclude that the results of the comparison between the monocrotaline model and the model combining monocrotaline and increased blood flow do not allow us to state that one is definitely superior to the other and therefore recommend one over the other as the standard for future studies. Because of its ease of use, the monocrotaline model is still more widely employed, despite its limitations in mimicking the pattern seen in human PAH, as was confirmed in our study. Nevertheless, studies whose primary objective is to evaluate RV hypertrophy can benefit from the combination of monocrotaline and increased blood flow.

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