Pulmonary vessel casting in a rat model of monocrotaline-mediated pulmonary hypertension

Zhongkai Zhu[1,2,3,*], Yifan Wang[1,2,*], Amy Long[1,2], Tianyu Feng[4], Maria Ocampo[1,2], Sunny Chen[1,2], Haiyang Tang[5,6], Qiang Guo[7], Richard Minshall[8,9], Ayako Makino[10] , Wei Huang[3] and Jiwang Chen[1,2]

[1]Division of Pulmonary, Critical Care, Sleep and Allergy Medicine, Department of Medicine, University of Illinois at Chicago, Chicago, IL, USA; [2]Center for Cardiovascular Research, University of Illinois at Chicago, Chicago, IL, USA; [3]Department of Cardiology, The First Affiliated Hospital of Chongqing Medical University, Chongqing, China; [4]Key Laboratory of Medical Diagnostics, Ministry of Education, College of Laboratory Medicine, Chongqing Medical University, Chongqing, China; [5]Collage of veterinary Medicine, Northwest A & F University, Yangling, Shaanxi, China; [6]State Key Laboratory of Respiratory Guangzhou Medical University, Guangzhou, China; [7]Division of Critical Care Medicine, Department of Medicine, the First Affiliated Hospital of Soochow University, Soochow, Jiangsu, China; [8]Department of Anesthesiology, University of Illinois at Chicago, Chicago, IL, USA; [9]Department of Pharmacology, University of Illinois at Chicago, Chicago, IL, USA; [10]Department of Medicine, University of California, San Diego, La Jolla, CA, USA

*The first two authors equally contributed to this manuscript

Abstract

Pulmonary hypertension is a chronic vascular disease characterized by pulmonary vasoconstriction and pulmonary arterial remodeling. Pulmonary arterial remodeling is mainly due to small pulmonary arterial wall thickening and lumen occlusion. Previous studies have described intravascular changes in lung sections using histopathology, but few were able to obtain a fine detailed image of the pulmonary vascular system. In this study, we used Microfil compounds to cast the pulmonary arteries in a rat model of monocrotaline-induced pulmonary hypertension. High-quality images that enabled quantification of distal pulmonary arterial branching based on the number of vessel bifurcations/junctions were demonstrated in this model. The branch and junction counts of distal pulmonary arteries significantly decreased in the monocrotaline group compared to the control group, and this effect was inversely proportional to the mean pulmonary artery pressure observed in each group. The patterns of pulmonary vasculature and the methods for pulmonary vessel casting are presented to provide a basis for future studies of pulmonary arterial remodeling due to pulmonary hypertension and other lung diseases that involve the remodeling of vasculature.

Keywords

Introduction

Pulmonary hypertension (PH) is a severe and progressive disease characterized by sustained elevations in pulmonary artery (PA) pressure, defined hemodynamically by a mean pulmonary artery pressure (mPAP) ≥ 25 mmHg at rest that can lead to an increase in pulmonary vascular resistance and right heart failure.[1,2] Pulmonary vascular network and blood flow have been found to be different between patients with PH and healthy subjects.[3,4] Adventitial fibrosis, medial hypertrophy, and intimal thickening all contribute to the

progression of pulmonary arterial remodeling (PAR). PAR is mainly due to small pulmonary arterial wall thickening and lumen occlusion.[5] Plexiform lesions are also hallmark features of severe PH observed in humans.[6] Thus, high-quality visualization and quantification of vascular

Corresponding author:
Jiwang Chen, University of Illinois at Chicago, 909 South Wolcott Avenue, COMRB 3210, MC937, Chicago, IL 60612, USA.
Email: chenjw@uic.edu

changes associated with development of experimental PH may aid in the detection and understanding of disease pathogenesis.

Many imaging techniques have been developed to help visualize pulmonary vascular networks and organ morphology in rodent models of lung disease.[7,8] These methods include computed tomography, vessel angiography, and vessel casting.[9–14] The former two approaches are technically demanding and only a few detailed protocols are available. Vessel casting is a simple and effective method to examine the angioarchitecture of normal and pathological tissues. Vascular casting precisely reflects the structure of blood vessels and allows for observation of the arrangement of arterial networks and the microangioarchitecture of terminal capillaries, where waste and nutrients are exchanged before blood returns via the venous circulation.[15] In rodent models of PH, acquisition of high-quality pulmonary vessel images and image quantification remains technically challenging. To date, there has been no systematic report describing a method for observing fine details of the pulmonary vasculature in the rat model of PH using a casting method.

In this study, we have developed a simple and reproducible procedure to image the entire pulmonary vasculature in each lobe of the rat lung and quantify the changes that occur during monocrotaline (MCT)-induced PH.

Materials and methods

Reagents

MCT (Cat.# C2401) was purchased from Sigma-Aldrich. Microfil (MV-122 Yellow) was purchased from Flow Tech, Inc.

Models of PH

All experiments were approved by the Ethics/Animal Care Committee of the University of Illinois at Chicago. Male Sprague Dawley® rats (180–200 g, strain 400, Charles River Laboratories) were used in this study. The numbers of rats are detailed in each figure.

In the MCT-induced PH model, MCT was dissolved in 0.5 N HCl to 200 mg/ml, neutralized to pH 7.4 with 0.5 N NaOH, and then diluted with sterile water to 60 mg/ml. One dose of MCT (60 mg/kg body weight) was subcutaneously injected to rats. Phenotypic characterization study was then performed four weeks after MCT injection. Food and water were provided ad libitum and the animals were checked once per day.

Hemodynamic measurement and lung tissue histology

After four weeks, the animals were anesthetized with an intraperitoneal injection of a ketamine/xylazine cocktail solution (1 ml ketamine (100 mg/ml) + 100 µl xylazine (20 mg/ml); inject 300 µl per 250 g body weight). A tracheal cannula was then inserted, and the animals were ventilated with room air using a TOPO™ dual mode rodent ventilator (D14-069, Kent Scientific) set to maintain respiration at 90 breaths/min and tidal volume at 8 ml/kg body weight.[16] To measure right ventricular systolic pressure (RVSP) and mPAP, the chest of the rat was opened. A Millar pressure transducer catheter (SPR-869NR) was used for RVSP and mPAP measurements. RVSP was measured while a consistently stabilized pressure wave was shown after the transducer was plugged into RV and then directly moved forward into PA for mPAP measurement. At the end of the experiments, the hearts and lungs were perfused with phosphate-buffered saline (PBS) for blood removal. Fulton index, or the weight ratio of the right ventricle divided by the sum of left ventricle and septum (RV/(LV + S)), was measured and calculated to determine the extent of right ventricular hypertrophy (RVH). Lung tissues were fixed in 10% formalin for 24 h and then further processed for paraffin sectioning. The paraffinized lung tissue sections were used for hematoxylin–eosin (H&E) staining and the images were scanned by Leica Aperio AT2 (Leica Biosystems). Approximately 2 mm from the outer edge of the lung, distal pulmonary arteries (500 in number, diameter < 100 µm) were counted and their properties were quantified using an Aperio ImageScope system.

Pulmonary artery casting

The procedure for PA casting as described previously[14] was followed in this study. Heparin (1000 UI/kg) as an anticoagulant was injected intravenously, 10 min before anesthesia. The rats were anesthetized using a ketamine/xylazine cocktail and after removal of the anterior chest wall, a P-50 tube was inserted and kept in the right ventricle via a needle (25 G) for perfusion with PBS which drained from the left atrium. The lungs were perfused under a pressure of 12 cmH₂O which was sufficient to clear all blood as evidenced by the lung tissue turning white. During PA perfusion, freshly dissolved Microfil polymer mixture (MV compound: MV diluent: MV agent = 5:5:1) was instilled via 25 G needle inserted into the PA from the incision through the RV wall via a syringe pump (NE-1000, 0.73 µl/h). The Microfil mixture was gently infused into the PA under a dissecting microscope until it reached to the terminal branches of PAs and then stopped within 2–3 s. The lungs were then kept at room temperature for approximately 1 h while covered with a wet paper towel to avoid desiccation of the lungs. At the end of the experiment, the dissected lungs and hearts were rinsed in PBS for 10–15 min at room temperature or overnight at 4°C. They were then dehydrated in a series of ethanol solutions (50%, 70%, 80%, 95%, and 100%; 2 h each). After dehydration, the lungs were put into a methyl salicylate (Sigma-Aldrich) solution. When the lungs became translucent and the Microfil compound was clearly visible, they were photographed using a Stereo microscope (SKU: SM-7B-FRL, AMScope) with a

camera (SKU: WF200, AMScope) and transilluminator (TW-43 white light) to capture the pulmonary arterial vasculature of each lung lobe. A different microscope (BZ-X700, KEYENCE, DPI: 72 pixels/in) was used for viewing the end-branches of each lobe. The end-branch pulmonary vessel images were quantified by Image J to calculate numbers of branches and junctions in the pulmonary arterial networks.

Lung vessel casting image quantification

The following detailed protocol was used for rat lung vessel casting image quantification in this study:

1. Open the image with Photoshop
2. Create a new layer by clicking ctrl + shift + N
3. Name the new layer "Outline" and click "OK"
4. Select the new layer "Outline"
5. Click "Brush Tool"
6. Adjust the size of the brush to 5 pixel
7. Change the brush color to red
8. Careful trace and outline the desired area of branches (the outer edge of the lung)
9. Click "Image"
10. Click "Adjustment"
11. Click "Hue/Saturation"
12. Adjust "Lightness" to −100 and click "OK"
13. Select the bottom layer "Background"
14. Click "Eraser Tool"
15. Adjust the size of the eraser accordingly and clean the traced area of branches
16. Right click on the layer "Outline"
17. Click "Merge Down"
18. Save as "STEP2" (with TIFF format)
19. Open "STEP2" with Image J
20. Click "Analyze" → "Set Scale"
21. Input 80 for "Distance in Pixels"
22. Input 300 for "Known distance"
23. Input "Micrometers" for "Unit of length" and click "OK"
24. Click "Analyze" → "Tools" → "Scale Bar"
25. Input 300 for "Width in Micrometers"
26. Select "Red" for "Color" and click "OK" to add size bar
27. Click "Freehand selection" tool
28. Use "Freehand selection" tool to closely circle the traced area of branches
29. Measure the area by clicking "Ctrl + M"
30. Record the measurement of the area
31. Click "Process" → "Binary" → "Make Binary"
32. Click "Process" → "Binary" → "Skeletonize"
33. Click "Command Finer Tool" and search for "Analyze Skeleton"
34. Select "Analyze Skeleton (2D/3D)" and click "Run"
    (a) This command requires a download from the official Image J website
    (b) Simply search for and download the plug-in named "Analyze Skeleton", then place the file in the Image J plugins folder and restart Image J
35. Click "Show the Result"
36. Save the file "Result"
37. Open the "Result" data with Excel
38. Calculate the "Sum" of "Branch Number" and record the number
39. Calculate the "Sum" of "Junction Number" and record the number

Statistical analysis

Statistical analysis of experimental data was performed using SigmaPlot 11.0 and GraphPad Prism 5.1 (GraphPad Software, Inc., La Jolla, CA). Lung casting image quantification was done using Photoshop and Image J software. Results are expressed as mean ± standard error of mean from at least three independent experiments. Student's t test and ANOVA were used to compare two and three groups, respectively. $p < 0.05$ was considered statistically significant.

Results

Pulmonary arterial casts in the rat model of MCT-induced PH

Images of Microfil arterial casts of each of the five rat lung lobes are shown in Fig. 1(a). Note the decreased density of the distal pulmonary vessels in the MCT group. The number of PA branches and junctions were in the band approximately 2 mm from the outer edge of every single lobe and quantified by Image J. Both the number of PA branches and junctions in the distal vessels significantly decreased in the MCT group ($p < 0.001$, Fig. 1c,d).

Hemodynamic and lung histology data in the rat model of MCT-mediated PH

mPAP in rats was measured using a Millar catheter system. As shown in Fig. 2, we observed significantly elevated mPAP values in the MCT-treated rats as compared to the vehicle control group (control vs. MCT: 20.16 ± 0.2 mmHg vs. 40.62 ± 0.45 mmHg, $p < 0.0001$). Further, images of H&E stained lung tissue sections were scanned using an Aperio ImageScope system were used to assess vascular occlusion of small PAs (diameter: 50–100 μm). These images revealed complete occlusion of 154 ± 7 distal PAs in the MCT group and 0 in the control group. Thus, PAR occurs in the rat MCT model of PH consistent with the corresponding images of pulmonary arterial casts shown in Fig. 1. Finally, RVH was also observed in the MCT group as compared to the control group (0.53 ± 0.02 vs. 0.24 ± 0.01, $p = 0.0074$) suggestive of pressure overload-induced right heart failure.
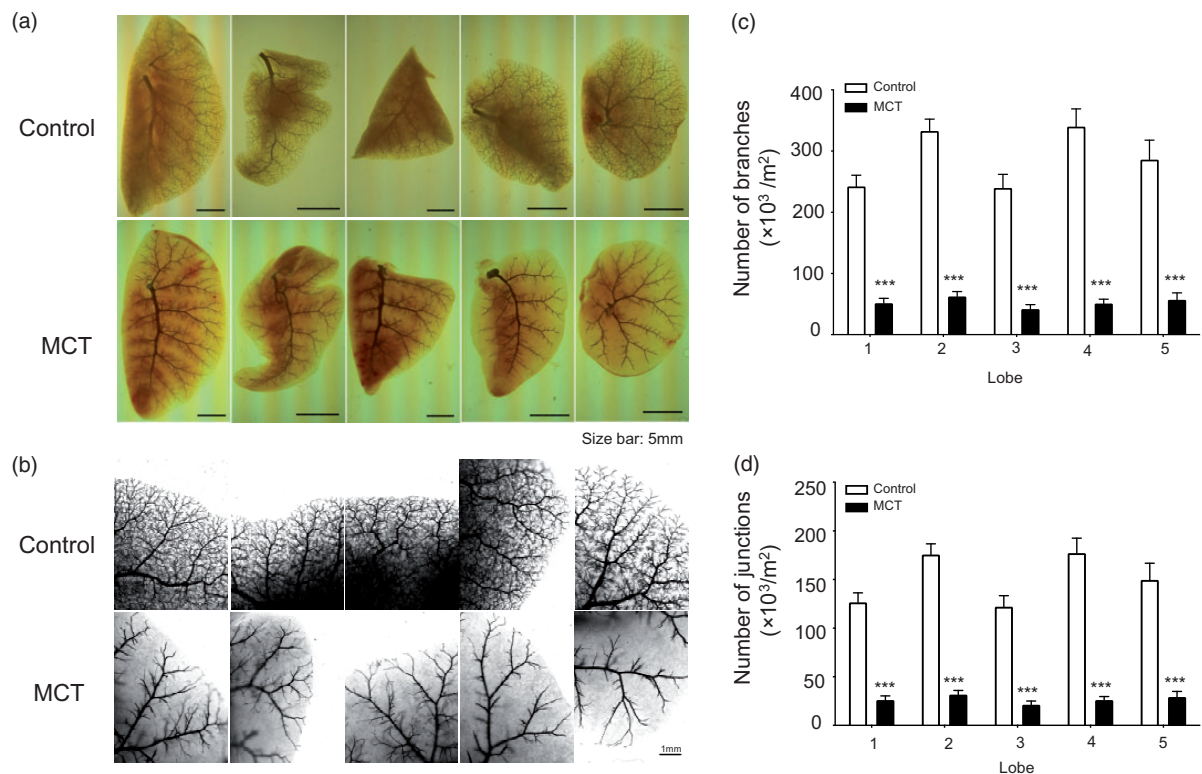
Fig. 1. Representative holistic pulmonary vessel images of five pulmonary lobes and image quantification in our rat model of monocrotaline (MCT)-mediated pulmonary hypertension. (a) Representative holistic pulmonary vessel images of five pulmonary lobes taken by a camera (SKU: WF200, AMScope) under a transilluminator (TW-43 white light); (b) distal pulmonary vessel images of five lobes taken by a microscope (BZ-X700, KEYENCE). (c,d) Number of pulmonary vessel branches and junctions in controls and MCTs. $N = 4$ in each group. $***p < 0.0001$.

Linear regression analysis

A linear regression analysis between mPAP and number of branches or junctions was performed in control and MCT rats which revealed a strong correlation ($r^2 > 0.8$) in this rat model of PH (Fig. 3). Furthermore, there was an inverse relationship between RVSP, branches and junctions, which was moderately correlated ($0.8 > r^2 > 0.6$) in this rat model of PH as shown in Fig. 4.

Discussion

In this report, we presented a simple and reproducible method to examine pulmonary vascular remodeling in the MCT-induced rat model of PH. Medial hypertrophy is a typical phenotype in PH and MCT treatment of rats has been widely used to induce and study PH. Here we clearly demonstrate obliteration of PA networks in all five lobes of MCT-treated hypertensive rat lungs. The detailed views of the entire pulmonary arterial networks and image analysis of distal PAs clearly show significant decrease in branches and junctions in distal pulmonary arteries in the MCT group. The results from casting data are consistent with the hemodynamic values and number of occlusive pulmonary vessels observed in this PH model. We found that MCT treatment was associated with a marked elevation in mPAP and RVH, as indicated by the RV/(LV + S) weight ratio, which both dramatically increased. This indicates that the higher pulmonary vascular resistance and afterload on the RV in the MCT-induced PH rat model lead to right heart hypertrophy.

Compared to previous studies,[17–19] we have the following innovations: first, we developed a simple imaging system for capturing the pulmonary vessel images of each rat lung lobe. Most of the previous pulmonary vessel casting studies were conducted in normal rats, and this direct comparison between normal and hypertensive rodents has not been performed; second, the distal pulmonary vessel images further analyzed by Image J were able to reveal reduced numbers of branches and junctions in the MCT model.

There are still some limitations to this study. First, we were able to analyze the distal pulmonary vessel branches, but not the entire pulmonary vasculature. This was because 1.25 magnification used to collect the vasculature of the entire lob was not of high enough resolution to be used for quantification from the thicker regions of the lobe. Second, we stopped filling the vasculature with Microfil when we observed that it reached the far end of pulmonary vessels. According to our experience, the volume of Microfil mixture needed for SD rats with 350–450 g body weight is
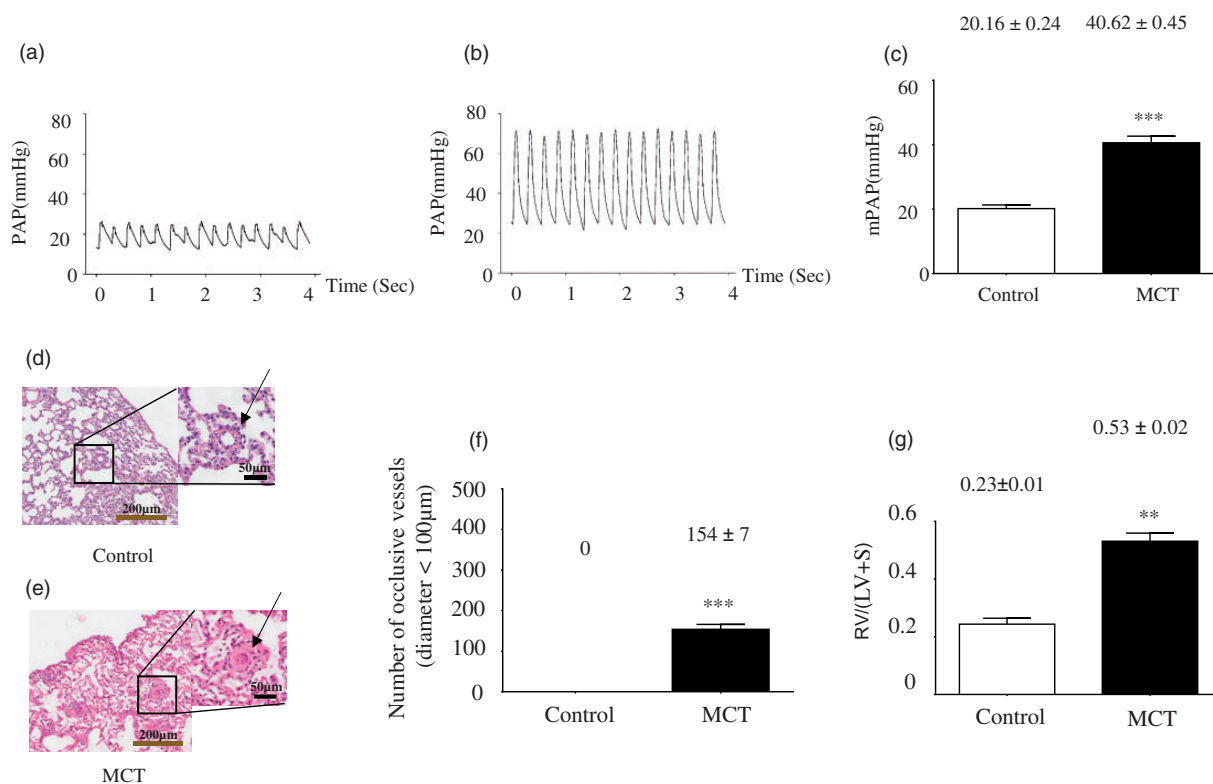
Fig. 2. Hemodynamic data of our rat model of MCT-mediated pulmonary hypertension. Panels (a -c): pulmonary artery pressure (PAP) tracings and summarized data of mean PAP (mPAP). Panels (d -f): representative lung images stained with hematoxylin -eosin (H&E) and number of occlusive pulmonary vessels in control and MCT groups (approximately 500 distal pulmonary arteries per rat were counted for each group, panel (g): ratios for RV/(LV ± S) in control and MCT group. N = 4 in each group. **p < 0.05; ***p < 0.0001 vs control.
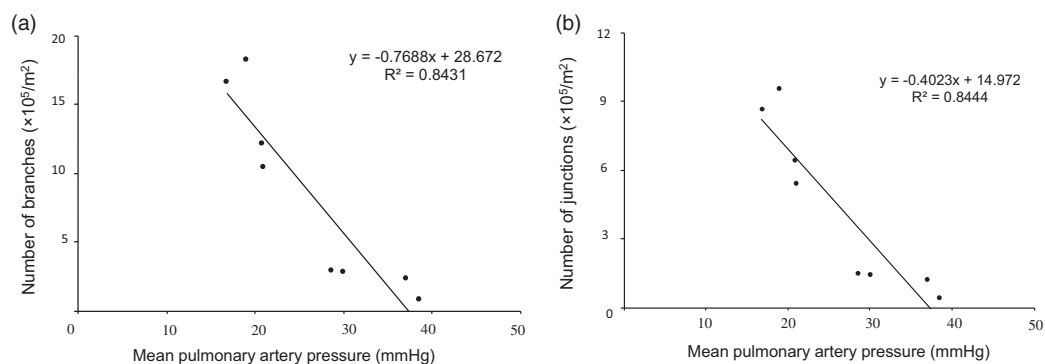

Fig. 3. Correlation between mean pulmonary artery pressure values and number of branches or junctions in five lobes measured by Image J in rats. N = 4 in each group.

approximately 300–350 µl. If more than the referenced volume is used, the mixture will go into the alveolar space and prevent imaging of only PA networks. Third, correlation between pulmonary vessel casting data and pulmonary vascular resistance could not be evaluated because rat cardiac output values were not measured in this study. Fourth, we did not measure the change of pulmonary vessel casting patterns in different stages of PH or with therapeutic treatment, and thus the sensitivity of our method could not be examined. We conclude that more advanced imaging systems to that may be able to tile together multiple high resolution images of pulmonary arterial networks, further optimization of the Microfil volume, correlation between rat pulmonary vessel casting data and their corresponding pulmonary vascular resistance, and measurement of pulmonary vessel casting patterns in different stages of PH progression warrant our further development of this application in terms of reproducibility and sensitivity.
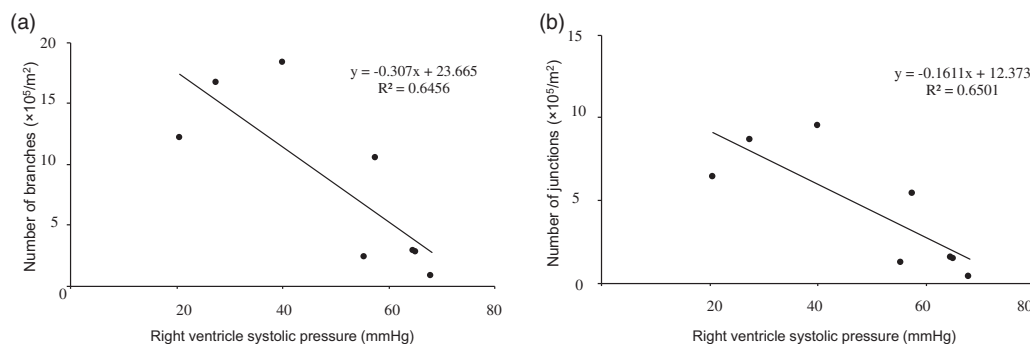
Fig. 4. Correlation between right ventricular systolic pressure values and number of branches or junctions in five lobes measured by Image J in rats. $N = 4$ in each group.

Taken together, we showed that casting of the pulmonary vasculature of MCT-treated rats can be used to assess vascular remodeling in PH. The distal pulmonary vessel images clearly demonstrate pulmonary vascular remodeling in rats with high mPAP. The innovative protocol and image quantification we presented here are important tools for understanding the pathology of PH and other diseases that involve remodeling of the pulmonary vasculature.

Conflict of interest

The author(s) declare that there is no conflict of interest.

ORCID iD

Ayako Makino https://orcid.org/0000-0003-1259-8604

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