Serial noninvasive assessment of progressive pulmonary hypertension in a rat model

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IN ANIMAL STUDIES OF PULMONARY hypertension (9), a rat model has often been used in which a single monocrotaline (MCT) injection induces pulmonary hypertension over a period of 3–5 wk. Performing right heart catheterization to measure right ventricular (RV) systolic pressure has been central to establishing the presence and quantifying the severity of pulmonary hypertension. Furthermore, gross and histological evaluation of heart and lung tissue has been performed to gain information as to the severity of disease development (7, 8, 10). However, these methods are limited by the number of measurements performed in the rat and in the ability to characterize rigorously the time course of pulmonary hypertension. Rat models are increasingly being utilized to evaluate the effectiveness of various therapeutic interventions for pulmonary hypertension. Because transthoracic echocardiography (TTE) is noninvasive and allows for multiple assessments of progressive pulmonary hypertension, it can be a useful complement to traditional methods of evaluating pulmonary hypertension in rats.

In humans, TTE is one modality for observing the progression of pulmonary hypertension. Although not the “gold standard” for identifying prognostic determinants in patients with pulmonary hypertension, qualitative parameters such as RV hypertrophy and dilatation, changes in pulmonary flow characteristics, right atrial (RA) enlargement, and tricuspid regurgitation (TR) are established echocardiographic features of pulmonary hypertension in humans. Furthermore, the TR velocity is routinely used to estimate pulmonary artery systolic pressure (PASP) (1). The TR velocity is used in the modified Bernoulli equation (4v2) to establish the RV-right atrial (RA) pressure gradient, which is an estimate of the PASP.

In rats, the use of TTE to study ventricular changes after an induced myocardial infarction is common (5). In 1988, Cottrill and colleagues (2) demonstrated that echocardiography could be used to assess RV hypertrophy in rats with pulmonary hypertension. However, owing to the technical limitations of echocardiography at that time, many currently established markers of pulmonary hypertension in humans have not yet been evaluated in animal models of the disease. In this study, we assessed the pulmonary artery (PA) flow waveform, the PA acceleration time (PAAT), RV free-wall thickness, RV end-diastolic dimension, and TR in a rat model of pulmonary hypertension. This study shows that TTE is applicable to rats in monitoring the progression of pulmonary hypertension.
METHODS

Animal treatment. Sixty-one male Sprague-Dawley rats weighing 225–250 g were used for this study. After four days of acclimatization, the animals were anesthetized with ketamine (100 mg/kg ip) and xylazine (7.5 mg/kg ip). Each animal’s chest was then shaved, and TTE was performed (see Echocardiographic technique). After the initial echocardiographic study, the rats were randomly divided into two groups. One group (control) was intraperitoneally injected with 0.25 ml of Dulbecco’s phosphate-buffered solution (D-PBS), and the other group (treatment) was injected with 60 mg/kg MCT. MCT was prepared at 60 mg/ml by mixing 400 mg of the compound with 6 ml of D-PBS and then adding 0.45 ml of 5 N HCl (to dissolve the compound) and 0.12 ml of 10 N NaOH (to neutralize the pH). The animals were then returned to their cages and given standard rat chow and tap water ad libitum for the duration of the study.

The study was extended to 44 days after treatment with MCT. From days 32 to 44, the MCT-treated animals that appeared clinically stressed were euthanized. Criteria used to assess the degree of illness of the animal included activity level, respiratory rate, and oral intake. These parameters were established with the approval of this institution’s animal care committee. For comparison, each time an MCT-treated animal was euthanized, a control animal was also euthanized. On day 44, all remaining animals were euthanized.

Echocardiographic technique. Transthoracic two-dimensional, M-mode, and Doppler imaging were performed in all animals using an Acuson C256 ultrasonographic system (Mountain View, CA) with a 15-MHz transducer. The 5-MHz transducer was also used for interrogation of the tricuspid valve. The transducer was maximally aligned to optimize endocardial visualization and spectral displays of Doppler profiles. M-mode and Doppler tracings were recorded at a sweep speed of 200 mm/s. M-mode measurements were performed according to recommendations of the Committee on M-mode Standardization of the American Society of Echocardiography. All studies were performed by an experienced sonographer who was blinded to the treatment group. Two observers, also blinded to treatment assignment, analyzed the images. All measurements represent the means of three cardiac cycles.

M-mode measurements. M-mode measurements of left ventricular (LV) wall thickness and cavity size were performed in the parasternal long-axis view at the level of the papillary muscles. RV free-wall thickness and end-diastolic cavity dimension were measured in the parasternal short-axis view just below the level of the aortic valve. This view was chosen because it offered the most consistent image of the RV free wall in this animal model.

Doppler imaging. Pulse-wave Doppler of pulmonary outflow was recorded in the parasternal view at the level of the aortic valve. The sample volume was placed proximal (3 mm) to the pulmonary valve leaflets and aligned to maximize laminar flow. In addition to characterizing the pulmonary outflow Doppler envelope, the acceleration time, velocity-time integral, and ejection time were measured. The velocity-time integral was obtained by tracing the outer edge of the pulmonary outflow Doppler profile. Acceleration time was measured from the time of onset of systolic flow to peak pulmonary outflow velocity. Ejection time was measured as the time from onset to completion of systolic pulmonary flow.

The tricuspid valve was interrogated for the presence of TR with color and continuous-wave Doppler in the apical four-chamber view so that the tricuspid and mitral valves could be clearly visualized (see Fig. 7A). If TR was observed, the transducer was aligned to achieve the maximal peak velocity. The systolic RV-RA pressure (P) gradient was calculated using the peak TR velocity (v) in the modified Bernoulli equation (AP = 4v²).

Right heart catheterization. An incision was made in the right side of the animal’s neck, and dissection was performed to expose the right jugular vein. A catheter was inserted into the vein and advanced into the right ventricle. Two types of catheters were used in this study. In experiments shown in all figures except Fig. 3B, a 0.04 × 0.023-in (OD by ID)-sized polyvinylchloride tube with a curved tip, which was filled with heparinized saline, was used. The tubing was connected to a Grass pressure transducer and linked to a Grass model 79 polygraph. In a second set of experiments (shown in Fig. 3B), a Millar 1.4-F Mikro-Tip catheter connected to a pressure-control unit and interfaced with a Gould signal amplifier and recorder was used. RV systolic pressure was recorded. This pressure is assumed to be equal to the PASP in the presence of a normal pulmonary valve. All right heart catheterizations were performed within 5 min of the echocardiographic examination; both procedures were completed within 30 min. As the right jugular vein was ligated after the catheterization was complete, no animal underwent the procedure more than twice.

Gross anatomic evaluation. After death, the animal’s heart was immediately dissected and the right ventricle and left ventricle plus septum were weighed. Additionally, the following weight ratios were calculated: right ventricle to body weight, left ventricle-body weight, and the right ventricle (left ventricle + septum).

Statistical analysis. All values are expressed as averages ± SD of the mean. Two-way ANOVA was used for RV thickness, RV dimension, and PAAT. After finding significant differences in RV thickness and changes in the AT between treatment and control groups, Tukey’s test was used for post hoc analysis. Pearson’s correlation coefficient was used to describe the relationship between the PASP estimated by TTE and the PASP measured by catheterization.

The interobserver and intraobserver variabilities were determined by the formula 2 SD of the mean. Measurements from four random time periods were compared. The interobserver variability was 10% for RV thickness, 3% for the AT, and 2.4% for TR. The intraobserver variability was 11% for RV thickness, 3.5% for the AT, and 3.3% for TR.

RESULTS

PA flow. To examine the usefulness of TTE in monitoring the development of pulmonary hypertension in rodents, we used a MCT-treated rat model, which is known to develop severe pulmonary hypertension 4 wk after a single-dose injection of MCT. Twenty-three rats were used in the initial study. Among them, 17 were injected with MCT and 8 were injected with D-PBS. TTE was performed on each rat on days 0, 7, 15, and 22 and every 2–3 days thereafter. Each rat also received a right heart catheterization at some time during the period of study (within 44 days), which was carried out within 5 min of TTE examination to correlate with the observed echocardiographic features.

Measurement of PA flow was feasible in all animals. All evaluations of PA flow were performed with a (maximal) sweep speed of 200 mm/s as slower sweep speeds may lead to a less accurate assessment of the
In addition to characterizing the PA waveform, the acceleration time, velocity-time integral, and ejection time were measured. We also screened for the development of pulmonary insufficiency, although at no point in the study was it observed. Two features of PA flow were found to be useful in estimating the progress of pulmonary hypertension in the rats: 1) change in the shape of the PA waveform, and 2) decrease of the PAAT. As shown in Fig. 1, the development of a PA midsystolic notch began on days 15–18 after MCT treatment, became more prominent on day 22, and was quite striking by day 37. On these days, the PASP of these rats was ~30 mmHg (day 15), 42 mmHg (day 22), and 55 mmHg (day 37), respectively. Figure 2 shows the change in the PAAT over time for both the control and MCT-treated rats. The decrease in the PAAT occurred initially between days 15 and 18, and it declined further with time. In comparing the value of the PAAT with the PASP measured by catheterization (Fig. 3A), we noticed that the PAAT correlated linearly ($r = 0.89$) with the PASP within the range of 33–20 ms (for PAAT) and 30–65 mmHg (for PASP). The linear relationship can be expressed as $\text{PASP} = 137.2 - 3.3(\text{PAAT})$, which provides a means of estimating the PASP with the PAAT. Outside this range, as the PAAT decreased from 38–40 to 33 ms, the PASP did not change. However, as normal rats have a PAAT of 38–40 ms (see Fig. 2), the decrease of the PAAT from 40 to 33 ms may represent changes in the pulmonary vasculature that are not reflected in the PASP. At PASPs > 65 mmHg, the PAAT did not continue to decrease as the PASP steadily rose. This phenomenon may be related to a reduced pulmonary flow volume secondary to the significant TR that develops at these pressures.

To determine whether the relationship between the PAAT and the PASP is altered by the type of catheter...
used for right heart catheterization, we performed a second study (n = 36 MCT-treated rats) using a Millar Mikro-Tip catheter to measure the PASP. The correlation between PAAT and PASP is shown in Fig. 3B. A linear correlation (r = 0.84) was found between the PAAT and the PASP in the range of 20–33 ms (for PAAT) and 30–65 mmHg (for PASP). The corresponding linear equation is PASP = 164 – 4.5(PAAT). The linear range in which the PAAT (32–20 ms) correlated with the PASP did not change based on the type of catheter used; however, the linear range of the PASP did change. This may reflect the fact that the PASP measurement obtained using the Mikro-Tip catheter is systematically lower (~5 mmHg) than that obtained with polyvinylchloride or polyethylene catheterization tubing. The discrepancy between the two equations suggests that a fixed formula relating the PAAT and the PASP requires empirical testing with echocardiography and right heart catheterization under specific experimental conditions.

**RV free-wall thickening and changes in end-diastolic dimension.** RV free-wall thickening was a consistent echocardiographic feature of pulmonary hypertension in the MCT-treated rats. As shown in Fig. 4, images of the RV free wall could be obtained at all stages of pulmonary hypertension in the rat. RV thickening was apparent on day 22 (Fig. 4C) and became more dramatic by day 37 (Fig. 4D). Figure 5 shows the changes in RV free-wall thickening over time in the control rats and the MCT-treated rats. An increase in the RV free-wall thickness of the MCT-treated rats was statistically significant at day 21 (P < 0.05). RV free-wall thickening continued in the MCT-treated animals through day 44 of the study, when the average thickness reached 0.14 cm.

Analysis of the weight of the rat hearts at the time of death also shows a significant RV thickening in the MCT-treated rats. Table 1 demonstrates that the ratio of RV weight to body weight was significantly higher in the MCT-treated group compared to the control group (1.27 ± 0.059 vs. 0.76 ± 0.050 mg/g; P < 0.001). Similarly, the weight ratio of the RV to LV plus septum was significantly higher (P < 0.001) in the MCT-treated group compared to the control group.

A trend toward RV dilation was present in rats treated with MCT, although it generally occurred late in the course of the disease and varied significantly among individual rats. As demonstrated in Fig. 6, RV dilation in the MCT-treated rats first occurred at day 28, although it never achieved a statistically significant increase compared with control rats. As RV dilation was both an inconsistent and late finding in rats with pulmonary hypertension, it did not prove to be a reliable marker of progressive pulmonary hypertension in the rat.

**Heart rate and systemic blood pressure.** We observed no difference in heart rate or systemic blood pressure between the control and MCT-treated rats during the study. The heart rate was assessed during all echocardiographic evaluations with the rats anesthetized with the ketamine-xylazine combination. The heart rates at day 0 were 263 ± 7 and 278 ± 8 beats/min for control and MCT-treated rats, respectively (P = not significant); at day 44, the heart rates were 250 ± 7 and 267 ± 20 beats/min for control and MCT-treated anesthetized rats, respectively (P = not significant). The systemic blood pressure was measured by carotid artery cannulation at days 0 and 44. The average systolic blood pressure readings were 119 ± 3 and 124 ± 4 mmHg at day 0 and 122 ± 5 and 120 ± 3 mmHg at day 44 for MCT-treated rats and controls, respectively (P = not significant).
The development of TR was a prominent, although late, feature of rats treated with MCT. The TR velocity is used in the modified Bernoulli equation \(4v^2\) to establish the RV-RA pressure gradient, which is an estimate of the PASP. Figure 7A shows the sampling position at which TR was screened using color-flow Doppler with a 15-MHz transducer. Trace TR was initially observed at day 28 with color-flow Doppler emerging at various time points between days 28 and 44. The TR jet could be confirmed and quantified with continuous-wave Doppler only after reaching a velocity of \(\geq 3\) m/s. Figure 7B is an example of the TR waveform obtained with continuous-wave Doppler using a 5-MHz transducer. The velocity of this tricuspid regurgitant jet was 4.3 m/s with a corresponding PASP (measured by catheterization) of 95 mmHg. Figure 8 shows the linear correlation \((r = 0.92)\) between the RV-RA pressure gradient and the PASP measured by right heart catheterization. The equation that describes this relationship is \(\text{PASP} = 44 + 0.79(\text{RV-RA gradient})\). Using only the RV-RA gradient \(4v^2\) to predict the PASP would systematically underestimate the PASP in rats. The reason for this underestimation is unclear at this point. This study also found that the TR was not quantifiable at a velocity \(< 3\) m/s or at pulmonary pressures \(< 65\) mmHg, even though trace TR was observed by color-flow Doppler. This difficulty in quantifying TR at low velocity could be related to the shape of the rat chest or the sensitivity of the transducer of Fig. 4. Right ventricular (RV) free-wall thickening and dilation as demonstrated by M-mode echocardiography. Rats were treated with MCT and studied at 0 (A), 7 (B), 22 (C), and 37 (D) days thereafter. Images of the RV were obtained from a parasternal short-axis view immediately below the level of the aortic valve. Note the increase in free-wall thickness at days 22 and 37 after injection of MCT compared with baseline.
the sonographic system used. Thus using TR velocity to estimate the PASP in rats is only suitable when severe pulmonary hypertension has developed. Assessing both the TR and PAAT provides parameters to estimate the PASP over a wide pressure range.

**Left ventricle.** No statistically significant change in either the LV free-wall thickness or end-diastolic dimension was observed during the study (data not shown). However, as the RV pressure approached that of the LV, interventricular septal flattening occurred and did reduce the LV dimension. This occurred very late in the course of the disease. On gross examination, the ratio of the LV plus septum weight to body weight was measured, and no statistically significant difference was noted between the MCT-treated group and the control group (2.315 ± 0.084 vs. 2.112 ± 0.064 mg/g; \( P = 0.075 \)).

**DISCUSSION**

This study examined the feasibility of applying TTE to monitoring the development of pulmonary hypertension in a rat model. Data showed that the echocardiographic images in rats are clear and consistent when obtained with a contemporary ultrasonographic system. Several echocardiographic features, such as changes in the PA waveform, the PAAT, RV free-wall thickness, and the development of TR, are found to be consistent with the trend of developing pulmonary hypertension in rats. These findings suggest that TTE could be a useful noninvasive method for investigating the time course of the development of pulmonary hypertension in rat models of the disease.

Central to the echocardiographic assessment of rats with pulmonary hypertension is the evaluation of PA

**Table 1. Body and ventricular weights**

<table>
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<th>Body Wt, mg</th>
<th>RV Wt/BODY Wt, mg/g</th>
<th>(LV + Septum Wt)/Body Wt, mg/g</th>
<th>RV Wt/(LV + Septum Wt), mg/g</th>
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<tr>
<td>Control</td>
<td>391 ± 7</td>
<td>0.756 ± 0.050</td>
<td>2.315 ± 0.084</td>
<td>0.325 ± 0.025</td>
</tr>
<tr>
<td>MCT</td>
<td>346 ± 6</td>
<td>1.274 ± 0.059</td>
<td>2.112 ± 0.064</td>
<td>0.603 ± 0.022</td>
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<td>( P ) value</td>
<td>&lt;0.001</td>
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Values are means ± SD from 15 monocrotaline (MCT)-treated and 8 control rats. Rats were treated and euthanized by day 44. Body wt, right ventricular (RV) wt, and left ventricular (LV) wt plus septal wt were measured.

Fig. 7. Tricuspid regurgitation (TR) velocity. Rats were treated with MCT and the development of TR was monitored with echocardiography for 44 days. Tricuspid valve was interrogated for the presence of TR in the apical four-chamber view with color and continuous-wave Doppler. If TR was observed, the transducer was aligned to achieve the maximal peak velocity. An example of the TR velocity obtained with a 5-MHz transducer using continuous-wave Doppler (A). An example of the view from which we recorded the TR velocity (B). RV, right ventricle; LV, left ventricle; RA, right atrium.
The changes in the PA waveform and the PAAT are found to be an early event in the development of pulmonary hypertension in rats. The initial change in these features occurred at a PASP of 30 mmHg (by fluid-filled catheter measurement) or 25 mmHg (by Millar Mikro-Tip catheter measurement). The correlation of the PAAT and the PASP is found to be linear within the PASP range of 30–65 mmHg (measured using a fluid-filled catheter) or 25–65 mmHg (using a Mikro-Tip catheter) and can be expressed by the equations PASP = 137.2 – 3.3(PAAT) or PASP = 164 – 4.5(PAAT), respectively. From the data collected in this study, the accuracies of using these equations to calculate PASP from the PAAT are ±3 and ±5 mmHg, respectively. However, it should be emphasized that these equations are not meant to be used universally. Changing the experimental conditions or the model, such as the animal species, anesthesia condition, sampling position for the PAAT measurement, or catheterization method, could affect the equation, and it is necessary to establish the slope and interception of the equation empirically under specific conditions.

Wide variations in heart rate can affect the measurement of PAAT. In this study, no significant difference was found between the heart rates of the control and MCT-treated rats. However, when the heart rate in rats decreased below 200 beats/min (as can occur with overanesthetization or by using a different anesthetic combination), the PAAT lengthened despite elevations in PASP, which makes the PAAT an unreliable estimate of PASP in this situation.

In the clinical use of TTE to assess pulmonary hypertension, electrocardiogram-based timing of cardiac events is critical. The RV preejection period and the preejection period-acceleration time ratio have been found to correlate with increasing PASPs (3, 4, 6, 9). However, in rats, the preejection period did not lengthen as the PASP increased. This may be due to the fact that the rat RV is so thin that it does not greatly affect (even when hypertrophied) the conduction system of the heart. As a result, the acceleration time proved to be the measurement that most closely correlated with increasing PASPs in this rat model.

TR velocity, as assessed with TTE, is routinely used in the clinical evaluation of patients with pulmonary hypertension. In this study, TR proved to be a useful parameter to estimate pulmonary pressures at PASPs > 65 mmHg, a range in which the PAAT does not correlate with the PASP. Unlike clinical observations, the initial detection of TR in rats by color-flow Doppler occurred at relatively high PASPs (~45 mmHg). This TR is quantifiable by continuous-wave Doppler only at PASPs > 65 mmHg. We used both the 15- and 5-MHz transducers in an attempt to detect TR at PASPs < 45 mmHg but were unsuccessful. The relatively late observation of TR could be due to two possibilities. First, the TR simply may not have been present in rats until relatively high pulmonary pressures had been achieved. Second, the system used here may not have been able to detect the TR jet at slower velocities, which could be due to the small rat heart, the shape of the rat thorax, or the sensitivity of the equipment used for small-animal research. These difficulties in measuring TR also may explain why the use of the RV-RA gradient alone as an estimate of pulmonary pressure systematically underestimated the actual PASP.

Although the PAAT and the development of TR can be used to estimate the PASP in rats, the PA waveform change and the RV free-wall thickening are qualitative echocardiographic features of progressive pulmonary hypertension in this model as well. Regarding RV free-wall thickening, we did not correlate the measurements obtained with TTE with gross analysis of the rat heart. Therefore, the differences in the values of the RV free-wall thickness for MCT-treated rats and controls only demonstrate a trend toward the development of RV hypertrophy in the MCT-treated rats and should not be viewed as absolute values.

We have established the practical utility of TTE for the serial noninvasive assessment of pulmonary hypertension in a rat model. Confirming the relationship between PASP and PAAT or TR jet velocity by invasive methods is essential to define the quantitative accuracy of the method in a given model system. Having done so will enable the investigator to address the natural history of (rodent) models of pulmonary hypertension or the efficacy of potential therapeutic interventions.

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