Validation of a Pressure Diameter Method for Determining Modulus and Strain of Collagen Engagement for Long Branches of Bovine Pulmonary Arteries

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Recent studies have shown that capacitance measurements of large arteries provide better prognosis and diagnosis than tests of resistance alone in pulmonary hypertension (Mahapatra et al., 2006, "Relationship of Pulmonary Arterial Capacitance and Mortality in Idiopathic Pulmonary Arterial Hypertension," J. Am. Coll. Cardiol., 47(4), pp. 799–803; Reuben, 1971, "Compliance of the Human Pulmonary Arterial System in Disease," Circ. Res., 29, pp. 40–50). Decreased arterial capacitance causes increased load to the heart and is the direct result of increased stiffness and elastic modulus of the arterial wall. Here, we validate a pressure-diameter (PD) method for comparing the elastic modulus and collagen engagement for post-hilar pulmonary arteries with a large range of arterial diameter. The tissue mechanics of the post-hilar arteries are not well-characterized in pulmonary hypertension. It is believed that future studies with this method will provide useful insight into the role of passive tissue mechanics of these arteries in the pathophysiology of pulmonary hypertension, eventually improving clinical diagnosis, prognosis, and treatment. Post-hilar pulmonary arteries, excised from healthy and hypertensive calves and healthy cows, were inflated over a range of 0 [mm Hg] to 110 [mm Hg] in an isolated tissue bath. Internal pressure was recorded with an electric pressure catheter. Artery diameter and longitudinal stretch were recorded photographically. Stress-strain data curves were extracted using Lamé's law of thick-walled tubes. Radial strips were removed from each section and tested in a uniaxial (MTS) tester for validation. Both the elastic modulus and collagen engagement strain were similar to results obtained by more traditional means. The average difference between measured values of the two methods for collagen engagement strain was 3.3% of the average value of the engagement strain. The average difference between the measured values of the two methods for modulus of elasticity was 7.4% of the average value of the

modulus. The maximum, theoretical, relative error for the stress determined with the PD method was calculated at 20.3%. The PD method proved to be a suitable replacement for uniaxial strain tests in comparing collagen engagement strains. The method allowed faster testing of tissues of multiple diameters, while removing the effect of end conditions. The PD method will be of further utility in continued study of tissue mechanics in pulmonary hypertension studies. [DOI: 10.1115/1.4006686]

Keywords: pressure-diameter, pulmonary hypertension, collagen engagement, modulus of elasticity

Introduction

Pulmonary hypertension (PH), defined as a mean pulmonary arterial pressure above 25 [mm Hg], leads to increased loading on the right ventricle of the heart, high rates of morbidity, and death [3].

Recent studies have shown that the progression of the disease is related to decreased compliance of the arterial wall. Increased arterial wall stiffness and elastic modulus, caused by tissue remodeling and collagen engagement, cause increased impedance and decreased compliance of the pulmonary arterial tree [1,2]. This leads to increased loading on the right ventricle [4] and other detrimental effects.

Tissue mechanics of the pre-hilar pulmonary arteries have been well-documented [5,6], providing significant insight into the role of the passive tissue mechanics of these arteries in pulmonary hypertension. As conduit arteries can be classified as arteries of diameters greater than 1 [mm] [7], these studies have not quantified the mechanics of 37% of the conduit volume [8]. Because compliance is a function of the entire bed of conduit arteries, the tissue mechanics of the post-hilar arteries should also be studied to truly understand the pathophysiology of arterial stiffening in pulmonary hypertension, which would require hundreds of tests using traditional testing methods.

The goal of this study is to provide validation of a PD measurement of long, post-hilar pulmonary artery branches, as a method of comparing the strain of collagen engagement and the elastic modulus for multiple generations of the pulmonary artery, between different animal groups. It is believed that the PD method will provide an improvement over uniaxial stress-strain measurements of individual segments by improving the rapidity of testing and limiting the effect of end constraints, while maintaining a similar level of accuracy.

Methods

Dissection. The main branches of left and right post-hilar pulmonary arteries were isolated from five adult cows and three neonatal calves from both of the largest two lung lobes. Dissection was performed from the hilum and moved distally until the outer artery diameter reached approximately 3 [mm]. At each branch point, the largest diameter branch was followed for dissection and separated from the smaller branches, which were not isolated. Any loose connective tissue was removed. In this way, a continuous section of artery was removed from each lung lobe with a range of diameters from the largest diameter artery in the lobe to a smallest outer diameter of approximately 3 [mm]. All arteries were tested within 72 h of animal sacrifice, and tissues were stored in calcium and magnesium-free NaCl-PBS (0.01 [mol/l]; ionic strength 0.15; pH 7.14).

PD Method Inflation. After isolation, longitudinal sections were chosen between each major branch and marked with two small dots (diameter = 1–3 [mm]) of Loctite 280 (Henkel Corporation, Westlake, OH) black glue. The two dots per section were placed in approximately the same location circumferentially and separated longitudinally by approximately 10 [mm]. Sections

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Fig. 1 Image of excised pulmonary artery branch showing placement of Loctite dots

were chosen to be longitudinally distant from major branch points, while avoiding proximity to holes, branches, or tears. Figure 1 is an image of the arterial marking. A thin latex liner (0.0685 [mm] wall thickness; 10.2 [cm] circumference) was inserted along the lumen of the arterial section to prevent pressure leaks through the branches. A liner diameter larger than the tissue diameter was chosen to avoid stretch or load support in the liner. The proximal end of the artery was cannulated and secured with umbilical tape. A thin nylon line was fed through the artery to maintain the arteries' longitudinal alignment in the tissue bath during testing. This string was attached to the cannulation point and the same point on the opposing wall of the tissue bath. The tissues were submerged in calcium and magnesium-free NaCl-PBS (0.01 [mol/l]; ionic strength 0.15; pH 7.4). The distal end of the artery was closed with a suture, preventing pressure leaks while allowing longitudinal movement. Each arterial section was then inflated with water to an internal pressure of approximately 110 mm Hg (gauge). A *Millar Mikro-Tip SPC-350* (Millar Instruments Inc., Huston, TX) catheter, just upstream of the cannulation point, measured pressure. Figure 2 provides a basic schematic of the testing setup. Prior to any pressure diameter measurement, each tissue was subjected to 9 cycles, in which the internal pressure was increased from atmospheric pressure to 110 [mmHg] (gauge) and then released. In this way, the artery was prestrained and any hysteresis was reduced [9].

Immediately following artery prestrain, pressure diameter measurements were made. Increasing the head height applied through the artery cannula gradually increased internal pressure. This was done using a constant flow DC pump with a flow control valve used to limit the rate of change in the height of the pressure head. As the internal artery pressure was increased, digital photographs

Fig. 2 Basic schematic of testing setup

of the entire tissue lengths were taken with a *Cannon Rebel XTli* (Canon U.S.A., Lake Success, NY) with an *EFS 55-150mm* (Canon U.S.A., Lake Success, NY) zoom lens at a rate of 1.7 [Hz]. A ruler with inch markings was also placed in the imaging plane of the artery, which enabled determination of pixel length. The camera shutter was triggered by a transistor driven switch signaled by a 1.7 [Hz], 5 [V] peak-to-peak square wave signal. In this way, the shutter was released at every moment the voltage wave reversed polarity from negative to positive. By simultaneously recoding the voltage wave and the pressure signal from the catheter on a 12-bit data acquisition system, the internal pressure at the time of each image was determined. The images were used to measure the outer diameter of the artery and the longitudinal distance between the two dots in each section. Thus, the result was a pressure-diameter data point and longitudinal strain for each image. Figure 3 is a typical pressure diameter plot.

After completion of the pressure diameter test, the internal pressure was relieved and the tissue was removed from the bath. The tissue was then cut twice along the plane normal to the longitudinal axis of the artery in each section. This formed rings with a width of 1 [mm]-3 [mm] for each section. Using the same camera

Fig. 3 Typical pressure diameter curve

t_0 and t were the unstrained and strained wall thicknesses, respectively, and d_0 and d were the unstrained and strained longitudinal distance between the Loctite marks in each section, respectively.

Engagement Strain. For the purposes of comparison, collagen engagement points were defined for both the MTS method and the PD method. For both methods, a ninth order polynomial was fit to the stress-strain trace. After application of the polynomial fit, the collagen engagement point was defined as the point of maximum curvature of the polynomial within the range of the tested data points. The curvature was calculated as

$$\kappa = \frac{\sigma''}{(1+\sigma'^2)^{3/2}} \quad (8)$$

where the stress derivatives are taken with respect to strain.

Smoothing. Prior to polynomial fitting, data smoothing of the stress-strain trace was necessary for the PD method due to noise introduced by the edge (diameter) detection process. Although the use of automated edge detection for diameter measurement was typically very accurate, as shown below, it occasionally introduced noise to the stress-strain curve by improperly selecting the edge of the artery, due to tissue discoloration or the presence of connective tissue. This error appears as noise rather than a consistent offset for each sample, due to inconsistent orientation of the artery with changes in pressure and artery motion due to fluid flows within the tissue bath (caused by pressure leaks in the artery or artery liner). The data was smoothed using a moving average filter. Smoothing was conducted with the minimum span size to produce a smooth polynomial fit. The maximum smoothing span for all data sets used was a span of 7 [data points] and an average span of 5.3 [data points]. Figure 5 shows the original, unsmoothed stress-strain curve with a solid line and the smoothed data curve with a dashed line. This figure illustrates the effectiveness of the smoothing function for noise reduction while maintaining the shape of the curve.

Modulus. The modulus of elasticity was defined for both measurement methods as the slope of the stress-strain curve. The modulus was calculated at 30% strain for all tissue sections. This

strain level was chosen to maintain consistency, as data for this strain was present in all tissue data sets and was lower than the engagement strain for all tissues. The modulus was determined by applying a linear fit to the stress-strain curve between 27.5% strain and 32.5% strain using the least squares method. The modulus was then defined as the slope of the linear fit.

Error. Theoretical error was calculated for the stress and strain determined by the PD method. Precision error was defined as half of the resolution of the measuring device for each measurement. Random error was defined using a tissue that demonstrated typical deviation from the MTS method in the PD data set. For the majority of measurements, random error was defined as the standard error in a population of 5 sample measurements. For the diameter measurements, random error was defined as the standard error in a population of 10 sample measurements, as 10 measurement samples were taken in an effort to reduce noise in the diameter measurement during all tests. Unlike the other measurements, error in the diameter was the result of four different sources: random error, precision error, random error in the orientation of the longitudinal axis, and precision error in the orientation of the longitudinal axis. Total rotational error was defined as

$$E_D = \sqrt{D^2([1 - \cos(E_{pa})]^2 + [1 - \cos(E_{ra})]^2) + E_p^2 + E_r^2} \quad (9)$$

where E_D was the total error of the diameter due to longitudinal alignment error, E_{pa} was the precision error in longitudinal alignment (degrees), E_{ra} was the random error in longitudinal alignment, E_p was the precision error in diameter measurement, E_r was the random error in diameter measurement, and D was the measured outer diameter of the tissue.

Results

Modulus. The modulus of elasticity was determined for 35 tissue samples with one outlier removed, resulting in 34 samples for both the PD method and the MTS method. The average slope found was 118 [kPa], which represents typical values as reported by Lammers et al. [6]. The mean of the difference (MTS method – PD method) between the modulus found using both methods was determined as

Fig. 5 Smoothing function applied to typical stress-strain curve using PD method

Fig. 6 Bland-Altman plot for modulus of elasticity

8.7 [kPa]. The standard deviation for the differences between the two methods was determined as 32.7 [kPa]. A paired t-test did not show a significant difference between the results of the two tests at a 95% confidence interval. Figure 6 is a Bland-Altman plot comparing the modulus for the two methods. These plots are presented through the remainder of the paper, so we provide a brief explanation. The x-axis shows the mean value of both measurement methods for each data point. The y-axis is the difference between the measured values. The dashed line is the mean of differences between the two testing methods for each data point, and the dot-dashed lines represent $\pm$ two standard deviations of the differences. When two testing methods are similar, the mean line should be close to zero and the magnitude of the vertical distance between the mean line and each data point should be small in comparison to their value on the x-axis. Finally, there should be no obvious trends in the data points.

Engagement Strain. The engagement strain was determined for 31 tissue samples with one outlier removed, resulting in 30 samples for both the PD method and the MTS method. Four tissues were not included, because they were not strained to the point of collagen engagement during PD testing, as determined by the MTS method. The average calculated engagement strain for the tests of calves using both methods was 51.3%. This value compares well with a study performed by Lammers et al. [6], where engagement strains were reported as 49% and 51% for control and hypoxic calves for the main pulmonary artery. The mean of the differences of collagen engagement strain (PD method – MTS method), as determined by the two methods, was -0.0212% strain. The standard deviation of the differences of collagen engagement strain was 0.0417% strain. On the other hand, a paired t-test did show a significant difference between the results of the two testing methods ($P = 0.007$). Figure 7 is a Bland-Altman plot comparing the collagen engagement strain for the two methods.

Theoretical Error. Calculation of the theoretical error was described in the Methods section. The maximum relative error found for the selected tissue sample was found as 20.3%. Each error element was set to zero individually, and error was recalculated to determine which measurement contributed the most to the error. It was determined that the majority of the error is due to the

high precision error of the longitudinal length measurement. With the precision error of the longitudinal length set to zero, the remaining error is only 7.8%. It should be noted that the estimate of precision error in the longitudinal strain is likely a significant overestimate of the true error, because of the care used in selecting the center of the dots used to measure the longitudinal stretch. This assumption is supported by the random error in longitudinal strain, which was approximately 13% of the calculated error in precision. Figure 8 is a typical stress-strain curve calculated with the PD method with error bars plotted.

Ad Hoc Variance Testing. Variance testing was conducted on four tissue sections as described previously. The average standard deviation in the measured modulus of the four tissue sections was 11.6% of the mean modulus using the PD method and 17.6% of the mean modulus using the MTS method. Using a t-test and a 95% confidence interval, it was shown that these two means could not be assumed different. On the other hand, equivalence of the two methods was not established unless an equivalence interval equal to or greater than 38% of the average value of the standard deviation of both groups was assumed at a 95% confidence interval. Equivalence was established as described by Hatch [10].

Ad Hoc Longitudinal Variance Testing. Analysis was performed by defining the error in each longitudinal strain measurement as the difference between the measured longitudinal strain and the average longitudinal strain measured at the corresponding pressure and longitudinal location. The mean error found was 2.6% strain, and the standard deviation of the error was 3.1% strain. The maximum error found over 120 data points was 15% strain. When the error is applied to the calculation of thickness (Eq. (7)), the error in thickness is also 2.6% and 8.7% of the measured thickness for the mean error and the mean error plus two standard deviations of the error. These values also approximate the error in the calculated stress (Eq. (6)), if it is assumed that the diameter of the artery is significantly larger than the thickness.

Discussion

The results of the tests indicated that the PD method is comparably effective to the uniaxial test method for making comparisons

Fig. 7 Bland-Altman plot for collagen engagement strain

Fig. 8 Typical PD and MTS stress-strain curves with PD error bars

of the strain of collagen engagement. The mean of the differences between the engagement strains represented only 3.3% of the average engagement strain of both methods. This small value indicated little or no difference between the methods, and this difference was easily accounted for by the overall theoretical error calculated for the PD method. Furthermore, there was no apparent trend in the error data that would have indicated systematic error. The standard deviation of the differences between the engagement strains represented only 6.5% of the average engagement strain of both methods. This small standard deviation showed that not only was the mean of the differences small, but it was consistently small over the entire population of tissues tested. Even so, it was also shown that there was a significant difference between the two test methods for engagement strain using a paired t-test. We believe that this is not a significant detriment to the PD testing method, as the mean difference between the two methods is very small in comparison to the actual strain of engagement and remains consistent, as can be seen by the low standard deviation

in the difference between the two methods. Furthermore, we present this testing method as a means of rapidly comparing the engagement strains between different animal groups and not a method for determining the precise in vivo engagement strain.

Similarly, the results of the tests indicated the PD method was well-correlated to the uniaxial test method for determining the modulus of the elastin. The mean of the differences between the two methods was equivalent to 7.4% of the average modulus. This difference was not shown to be significant using a paired t-test.

Error calculations were also performed to demonstrate the effectiveness of the PD method. The maximum, relative, theoretical error in the stress measurement, in the range of stress normal for the PD method, was found to be 20.3%. Although this error was relatively high, a large portion of this error was due to the assumed precision of the longitudinal stress. Therefore, the use of an automated dot-tracing algorithm would greatly reduce the error when used in place of the manual dot selection. This method was not used, because the proximity of the two dots in each section

caused automated dot-tracing algorithms to often select the wrong dot. It is believed that an improved algorithm could solve this issue.

The tests performed showed the utility of the PD method for determining differences in the strain state of collagen engagement for long pulmonary arterial branches excised from healthy and hypertensive calves and cows. Further, the small average difference between the two methods indicates that the PD method was an effective predictor for changes in the elastic modulus of the elastin-dominant region of the stress-strain curve between animal groups.

The ad hoc longitudinal variance testing performed in this study indicated only small error based on the radial location of the placement of longitudinal strain markers. Although we do believe that the addition of multiple longitudinal strain measurements would increase the overall accuracy of the testing method, we believe that the spread in error found in the ad hoc testing indicates that the use of only a single measurement is acceptable based on the accuracy of the other measurements made in the study and the number of tissues tested in this study.

Overall, we believe that the validation of the PD method was a success. This method allowed a significant decrease in testing time and effort, in comparison to the MTS method, because sections of multiple different diameters were tested simultaneously and the data correlated well with data collected using MTS methods. Finally, the data collected in these tests showed the validity of lining the artery lumen with a latex liner to prevent pressure leaks during a PD test.

Limitations. Although the mean difference in modulus between the two methods was small, the standard deviation of the differences was found as 27.8% of the average modulus. We believe that, although this indicated a high degree of variation over the sample population, this could be the result of inconsistencies in the tissues and not error in the method. This was supported by the mutually high standard deviation shown in the ad hoc variation testing. Furthermore, there was no apparent trend in the error that would have indicated systematic error.

The PD method was also less effective than the MTS for testing localized changes in stiffness. The PD method applied consistent pressures over the entire section and should therefore have given a stress-strain curve that was representative of the average material mechanics for the entire section. The MTS was able to test very

specific circumferential strips. This was not believed to be useful for the majority of compliance studies in PH.

Finally, both the PD and MTS testing methods presented here did not match the in vivo, longitudinal stretch of the arteries. Because of inhomogeneity in the arterial wall material, the exact stress-strain characteristics of the tissue, in vivo, cannot be predicted exactly by tests with stress or strains prescribed only in the circumferential direction. It is for this reason that we present this methodology as a tool for comparing the material characteristics of multiple animals and not a method for predicting the true stresses and strains present in vivo.

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