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Biaxial mechanical behavior of bovine saphenous venous valve leaflets

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ABSTRACT

Chronic venous disease is caused by chronic venous insufficiency (CVI), which results in significant symptoms such as venous ulcers, ankle eczema, leg swelling, etc. Venous valve incompetence is a major cause of CVI. When the valves of veins in the leg become incompetent (i.e., do not close properly), blood is able to flow backwards (i.e., reflux), which results in blood pooling in the lower extremities, distal venous hypertension, and CVI. Current clinical therapies, such as surgical venous valve reconstruction and bioprosthetic venous valve replacement, are highly invasive and only moderately successful. This is due, in part, to the scanty information available about venous valve leaflet structure and mechanical properties. To date, only one previous study by our research group has reported on the mechanical properties of venous valve leaflet tissue, and specifically in the case of jugular vein valves. In this study, we conducted equibiaxial tensile tests on bovine saphenous vein valve leaflet tissues to better understand their nonlinear, anisotropic mechanical behavior. By stretching the valvular tissues to 60% strain in both the circumferential and radial directions, we generated stress-strain curves for proximal (i.e., those closest to the heart) and distal (i.e., those furthest from the heart) valve leaflets. Histology and collagen assays were also conducted to study corresponding leaflet microstructures and the biochemical properties of the tissues. Results showed: (1) saphenous venous valve tissues possessed overall anisotropic properties. The tissues were stiffer in the circumferential direction than in the radial direction ($p < 0.01$), and (2) saphenous venous valve tissues from the proximal end showed nonlinear isotropic mechanical properties, while those from the distal end showed nonlinear anisotropic mechanical properties. (3) Distal saphenous venous valve tissues appeared to be stiffer than proximal ones in the circumferential direction, $p = 0.04$ (i.e., inter-valvular variability), and (4) the collagen concentration showed a decreasing trend from the proximal to the distal end. This study focuses on highly relevant animal (bovine) tissues to develop test protocols, establish biomechanical structure-function correlations, and to provide data critical to the design of clinical prosthetic venous valves. To the best of the author's knowledge, this is the first study reporting the biaxial mechanical properties of saphenous venous valve leaflet tissues and thus contributes toward refining our collective understanding of valvular tissue biomechanics.

1. Introduction

Chronic venous insufficiency (CVI), the result of venous hypertension and venous valve incompetence, ranks among the most prevalent and economically costly vascular diseases in the US, afflicting up to 35% of adults (Zervides and Giannoukas, 2012). In 2014, the US health care cost of CVI was around one billion USD, with around 4.6 million working days lost per year due to venous-related disorders (Zervides and Giannoukas, 2012). The prevalence of CVI has been estimated to be twice as high in women as in men and increases with pregnancy (James et al., 1996; Sparey et al., 1999) and age (Beebe-Dimmer et al., 2005; Fan, 2005). By 2030, the American population over 60 years of age is expected to increase by ~40% relative to 2014, exacerbating this burgeoning health care crisis. CVI is considered a qualifying disabling condition by the US Social Security Administration (Committee, 2010). However, this increasing prevalence has not been met with commensurate increases in rehabilitation research, a situation attributed in part to the pernicious progression of CVI relative to other acutely lethal diseases, and also to the fact that the debilitating consequences of this chronic disorder are often overlooked (Milic, 2011). Indeed, unlike heart valve disease, CVI is frequently misdiagnosed, not diagnosed, or left untreated, with clinical presentation often written off as merely cosmetic or as an inevitable consequence of aging (George, 2012).

The pathophysiology of venous valve dysfunction is distinct from that of the semilunar heart valves. In both diseases, unidirectional blood flow is impaired, principally by regurgitant flow due to venous

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valve failure. In the heart, incompetent valves force the heart to work harder, culminating in congestive heart failure. In the legs, venous valve incompetence allows gravity to exert its full force on the column of blood between the right atrium and the veins of the thighs and legs, causing distal blood pooling and stasis (risking life-threatening clots), edema, inflammation, varicose veins, damage to the lymphatic system and skin, and, in advanced CVI, venous ulcers (i.e., recurrent, non-healing wounds) (Bergan, 2008). Yet, while CVI and its comorbidities harm quality of life to a degree approaching that of heart disease, comparatively little attention has been given to CVI or its unique venous valvular etiology.

While over 200+ studies have reported on the mechanical properties of the four mammalian heart valves and their leaflets, only a single study by our research group (Huang and Lu, 2017) has reported on the mechanical properties of venous valve leaflet tissue, and specifically in the case of jugular vein valves. Moreover, there is not even a single stress-strain curve published for saphenous venous valve leaflet tissue, despite the numerous venous valves (Edwards, 1940; Moore et al., 2011) and the over 2300 micro-venous valves (Meissner, 2005; Phillips et al., 2004) discovered to date. Therefore, the current study aims to comprehensively characterize the basic mechanical properties of normal animal saphenous venous valve leaflets.

Collagen forms the primary extracellular matrix (ECM) constituent of venous valve leaflets (Edwards, 1940). However, with the exception of semi-quantitative morphological measurements derived from transmission electron micrographs (Mouton et al., 2013), descriptions to date of normal venous valve ECM and pathological changes associated with CVI have been qualitative (Budd et al., 1990; Edwards, 1940; Ono et al., 1998). Quantitative biochemical measurements of saphenous venous valve leaflet ECM constituents do not exist in the literature, despite their importance for understanding venous valve incompetence and the progression of CVI. Presumably compensatory (Edwards, 1940) ECM synthesis has been microscopically co-localized with migrating and activated myofibroblast-like interstitial cells in diseased venous valves (Budd et al., 1990). As such, quantitative data on saphenous venous valve leaflet ECM composition (e.g., collagen) is important for understanding the mechanical responses of saphenous venous valve leaflets and their responses to CVI pharmacological interventions. Histological images of the saphenous venous valve tissues were also obtained to investigate microstructures in the tissue and their relationships with the mechanical properties. The objective of the study is to provide information on mechanical, microstructural, and biochemical properties of saphenous venous valve tissues, and this information is critical for understanding physiological and pathological venous valve tissue mechanics.

2. Materials and methods

Bovine saphenous venous valve (SV) tissues have been used as prosthetic valves in previous studies for CVI treatments (Reeves et al., 1997; Zervides and Giannoukas, 2012). Moreover, as the saphenous vein is located in the lower extremities where CVI usually occurs in the human body, studying SV properties would provide better information about the natural mechanical properties of the desired prosthetic valves. Bovine saphenous veins were obtained from the local abattoir. Tissues from cows (Holstein breed, female, 10+ yrs old, ~1250 lbs weight) used for meat production were shipped overnight on ice, ~24 h post-slaughter. Justifications for testing venous valves from bovine great saphenous veins include: (1) the relatively large vein size (~9–10 mm diameter (Buescher et al., 2005)) and (2) clinical relevance for prosthetic venous valves (Reeves et al., 1997; Zervides and Giannoukas, 2012). Saphenous vein samples were approximately 25 cm in length, with diameters of 9–10 mm (Fig. 1a), with plenty of soft tissue clinging to their surfaces. For saphenous vein samples, slight changes in vein diameter were also observed. The proximal ends appeared to be larger in diameter than the distal ends (Fig. 1a). As in the natural situation, the proximal end is closer to the thigh and the distal end is closer to the foot, this geometric change is considered physically reasonable.

Removing the soft tissue aided with the dissection process and made the leaflet samples easier to manipulate. After cleaning the surfaces of the vein samples, two pairs of tweezers were used to turn the veins inside out to expose the locations of the sinus and valves, and therefore prevent any accidental breakage of the valve leaflets during the dissection (Lu, 2016). During this process, one pair of tweezers was used to hold the vein, while another pair was used to grab the inside of the vein wall and pull it out gently. Once all the valve leaflets are exposed, the vein can be cut open longitudinally without breaking any leaflets. Since the vein samples were mostly around 25 cm in length, a series of venous valves (distal to proximal [toward right side of heart]) were collected from the bovine saphenous veins, such that differences in their properties from distal-to-proximal could be determined (i.e., inter-valvular variability). The longitudinal locations of all collected valves were recorded: due to the even distribution of valves along the length of saphenous veins, the portion within 12 cm of the first pair of valves from the proximal end was defined as “proximal.” The portion that was 12–25 cm from the first pair of valves was defined as “distal” (Fig. 1a). The dimensions of all isolated saphenous venous valve leaflets were recorded to provide general anatomical features, such as leaflet thickness, leaflet length L, sinus height H, and leaflet height h (Fig. 1b). By laying the vein flat on a board, the valve leaflets could be lifted carefully with a pair of tweezers. During this process, the tweezers should neither tear nor poke through the leaflet, which causes damage and may affect the native properties of the specimens. By holding the valve and cutting along the basal attachment, the semilunar leaflet can be isolated from the vein (Fig. 1b). Based on a well-established method (Billiar and Sacks, 2000; May-Newman et al., 2009; van Geemen et al., 2012), we measured the leaflet thickness 4–5 times with a 1010Z dial indicator pocket gauge (Starrett Co., Athol, MA, USA). In each of the vein sample, 6–12 pairs of valves were observed. During the dissection process, only bicuspid valves (i.e., two individual leaflets excised from the same venous valve apparatus) were observed in all saphenous vein samples.

Tissues from the central belly region of each leaflet were collected and oriented such that the orthogonal axes of the square specimens correspond to the circumferential (C) and radial directions (R), where collagen fibers aligned in the circumferential direction could be easily observed. A turnkey biaxial tester, the BioTester 5000 (CellScale, Waterloo, Ontario, Canada), equipped with two load cells (one per axis of loading; 10 N ± 0.02 N) was used for biaxial mechanical testing (Huang et al., 2012, 2014; Huang and Lu, 2017; Huang and Huang, 2015). To fully characterize the in-plane elastic mechanical properties, saphenous venous valve leaflets were subjected to a full range of biaxial and displacement-based loading protocols using the clamps (no shear; Fig. 1c-d). As the SV specimens were too soft, a piece of paper towel on top of a specimen was used as a support during the mounting process, to protect damage/tearing of the leaflets at the grips (Fig. 1c-d). When mounting the specimens, the circumferential direction (C) of the specimen was lined up with the x-axis of the BioTester, while the radial direction (R) was lined up with the y-axis. During the mounting process, the two clamps on the opposite position needed to be perfectly in line with each other to ensure no shear stress would be introduced during the testing process (Fig. 1c-d). The mounted samples were lowered into a temperature-controlled, 37 °C Hanks’ balanced saline solution (HBSS) bath. It was anticipated that preloading would release sample internal residual stresses (Huang et al., 2012, 2014; Huang and Lu, 2017; Huang and Huang, 2015) in addition to cutting the leaflets before testing. Briefly, samples were pre-loaded to 0.01 N, then pre-conditioned at 1%/sec to 30% strain for eight cycles to generate repeatable and reliable results, followed by a five minute recovery period (Huang and Lu, 2017).

Our study on the jugular vein valve tissues (Huang and Lu, 2017)

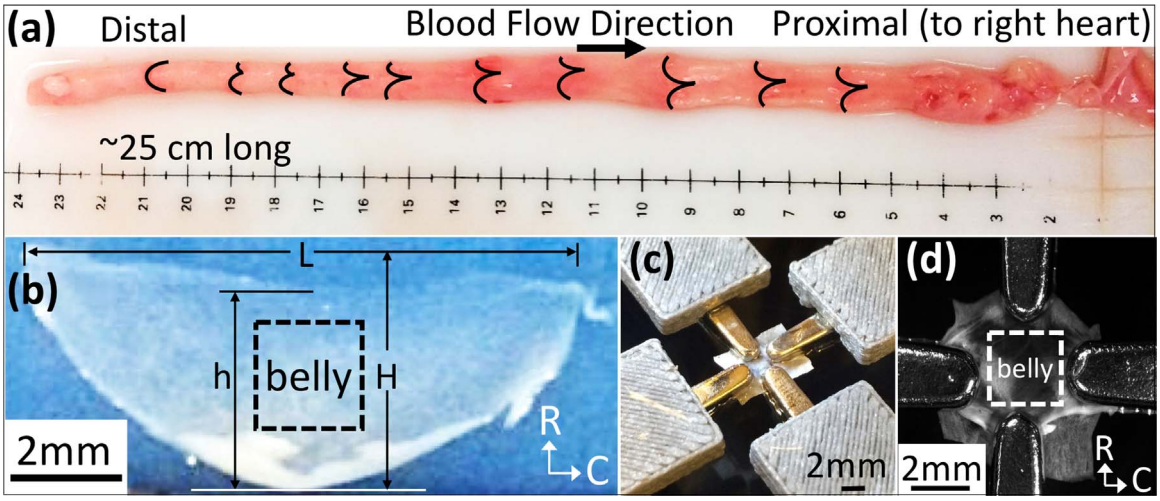

Fig. 1. (a) Bovine saphenous vein and (b) venous valve leaflet. C: circumferential, R: radial direction. 8 ± 2 valves every ~ 22 mm traced in black (a); intact leaflets partly visible because veins everted for excising. (b) Dimensions of valve leaflet are collected. Leaflet length L, sinus height H, and leaflet height h. (c) The customized clamps for small samples. (d) Dimensions of the dashed square are output for strain quantification.

has shown that tissues could be stretched up to 60% biaxially without sample breakage. Thus, the saphenous vein valvular tissue in the current study was also stretched to 60% at a rate of 1%/sec and lastly recovered to 0% strain. This information can be compared directly with data available on jugular valve leaflet tissues, i.e., valves at different locations inside the body. As a paper towel was introduced into the system due to mounting requirements, such support needed to be broken before collecting data from leaflet specimens. Therefore, an extra cycle of 60% stretch was included before the final testing cycle, in which the paper towel was broken and would no longer contribute to the force output. In summary, the mechanical testing protocol was determined to be performed equibiaxially with eight cycles of preconditioning to 30% strain at the same rate as the tensile test of 1%/s. After five minutes recovery after preconditioning, the specimen was stretched to 60% at the rate of 1%/s and at last recovered to 0% strain for two cycles. Grip-to-grip displacement and force data from leaflet specimens were collected during the last cycle.

Synchronized time lapse video for real-time monitoring and post-process analysis was provided by a charged-couple device (CCD) camera, which acquires images with a pixel resolution of 1280 × 960 at an acquisition rate of 10 Hz, with a lens focal length of 75 mm. Data logging capabilities ensure precise registration of axis loads, displacements, and image frames. Image tracking and analysis software (Labjoy, CellScale) was then used to review images collected during biaxial testing protocols. Sets of images were output as movie files (Huang et al., 2012, 2014; Huang and Lu, 2017; Huang and Huang, 2015).

Based on the sample dimensions and output data, corresponding nominal stress (i.e., First Piola-Kirchhoff stress) were calculated; the point-to-point distance from the clamps were recorded during the test via the actuators and the displacements changes were then calculated into true strains. Finally, stress-strain curves for the specimens were obtained. The peak tangent modulus was calculated in the relatively linear region of the stress-strain curve between 50% and 60% strain by subtracting the stress at 50% strain from the stress at 60% strain and then dividing the difference by the corresponding difference in strain magnitude (i.e., 10%). The peak stress was defined as the stress magnitude recorded at 60% strain.

Histological photomicrographs of saphenous venous valve leaflet samples were prepared as follows: Saphenous venous valve tissues from the proximal and distal groups were fixed in 10% (v/v) buffered formalin, processed by standard paraffin embedding, and stained with hematoxylin and eosin (H & E), Masson's Trichrome, and Verhoeff–Van Gieson (VVG). H & E stained collagen fibers are shown in pale pink and nuclei in blue or purple. Masson's Trichrome stained collagen appears blue, nuclei are black, and cytoplasm and keratin are red. VVG stained elastic fibers and nuclei both appear black, collagen is red, and cytoplasm is yellow. The histological slides were digitized as photomicrographs using a Zeiss Axiophot optical microscope (Carl Zeiss AG, Oberkochen, Germany) at 400× magnification using techniques adapted from published articles (Huang et al., 2012, 2014; Huang and Lu, 2017; Huang and Huang, 2015).

Additional 11 specimens from the proximal group and 11 from the distal group were collected for collagen assay. Collagen concentrations (collagen types I, III, & V) in the saphenous venous valve leaflets were determined via an assay kit (Sircol, Accurate Chemical & Scientific Corp., Westbury, NY, USA) using techniques adapted from our (Huang et al., 2012, 2014; Huang and Lu, 2017; Huang and Huang, 2015) and other papers (Engelmayr et al., 2005). Briefly, the specimens ($\sim 0.5 \times 0.5$ mm^2) from each venous valve tissue were frozen after dissection. The samples were weighed using an analytical balance (VWR, West Chester, PA, USA). Specimens were extracted in a solution of acetic acid (0.5 M; Sigma-Aldrich, St. Louis, MO, USA) and pepsin (1 mg/mL Pepsin A [P-7000]; Sigma-Aldrich, St. Louis, MO, USA) (Huang et al., 2012, 2014; Huang and Lu, 2017; Huang and Huang, 2015). One mL of collagen extraction solution was added to each tube and samples were placed on a vortex mixer (VWR, West Chester, PA, USA) for 120 h. The collagen concentration was quantified based on the precalculated collagen standard curve described in our previous work (Huang et al., 2012, 2014; Huang and Lu, 2017; Huang and Huang, 2015). Briefly, a collagen standard curve ($y = 0.0284x$, $R^2 = 0.9994$) was established using four absorbance values from the standards, where y is the absorbance value at 550 nm, x is the mass of collagen, and R is the correlation coefficient between x and y. The collagen mass of 22 samples were calculated by comparing absorbance values to those of the collagen standards via a spectrophotometer (Thermo-Fisher Scientific, Waltham, MA, USA). The collagen concentration of each sample was then calculated by normalizing to the wet weight of the individual collagen sample. This information could be directly compared with data available on jugular valve leaflet tissues (Huang and Lu, 2017), i.e., valves at different locations inside the body.

2.1. Statistics

Statistical analyses were conducted using JMP (SAS Institute Inc. Cary, NC, USA). Comparisons involving one factor (e.g., valve location on the distal-to-proximal axis) were conducted using one way ANOVA,

with Tukey's post-hoc test for significance ($p < 0.05$) of multiple comparisons. To verify the anisotropic properties of the valve tissues, paired *t*-tests were used to compare peak tangent moduli and maximum stresses in the circumferential and radial directions. Data are presented as the means ± standard deviations.

3. Results and discussion

Saphenous venous valves were obtained from bovine saphenous veins following eversion (i.e., turning inside-out), so as to facilitate identification of the leaflet margins and thereby mitigate damage. During the dissection process, different vein samples did not contain the same numbers of valves, from 6 to 12 pairs of valves were observed in each of the vein samples. Only bicuspid valves were observed in the saphenous vein samples, which is different from jugular vein samples (Huang and Lu, 2017; Lu, 2016) in which transition from primarily bicuspid valves on the proximal side of the jugular vein towards tricuspid valves on the distal side is observed. On average, the saphenous venous valvele aflet length L = 6.29 ± 1.86 mm, sinus height H = 2.98 ± 1.23 mm, leaflet height h = 2.72 ± 1.18 mm, and thickness = 30.45 ± 17.38 μm (Fig. 1b).

After averaging out the measured stress vs. strain for the saphenous venous valve leaflet samples under equibiaxial testing from a total of 6 veins, all of the specimens in the proximal (n = 18) and distal (n = 18) regions showed nonlinear mechanical properties with differences between the circumferential and radial directions (Fig. 2). In Fig. 2, three zones are observed: the toe region is between 0% and 25% strain stretching, the exponential region is between 25% and 50% strain stretching, and the linear region is between 50% and 60% strain stretching. The peak tangent moduli of elasticity of the tissues were then calculated for the linear region between 50% − 60% strain and the peak stress values at 60% strain were also collected. For the proximal tissue specimens, while the circumferential direction had a peak tangent modulus of 62.7 ± 34.9 MPa, which was 1.5 times larger than that of the radial direction, 41.6 ± 16.0 MPa, the differences did not reach statistical significance (top inset of Fig. 2). For the distal tissue specimens, the circumferential direction had a modulus of 92.1 ± 27.9 MPa, which was almost three times larger than that of the radial direction, 31.8 ± 13.9 MPa ($p < 0.01$) (Table 1). The difference for this mechanical property between the circumferential and radial directions was as large as that in the distal specimen. Based on statistical analysis, significant inter-valvular variability existed in the peak tangent moduli of elasticity in the circumferential direction ($p < 0.05$). Further, based on the stress-strain curves and peak tangent moduli of elasticity, a trend toward decreasing radial mechanical properties is visually observed from the proximal to the distal end (Fig. 2). Distal tissues appeared to possess larger peak tangent moduli of elasticity in the circumferential direction as compared with proximal tissues, yet the radial peak tangent moduli appeared to be quite similar. The leaflets appeared to have higher peak tangent moduli in the circumferential direction than in the radial direction, which indicated that given the same force level, the leaflets would be stretched unequally in the circumferential and radial directions.

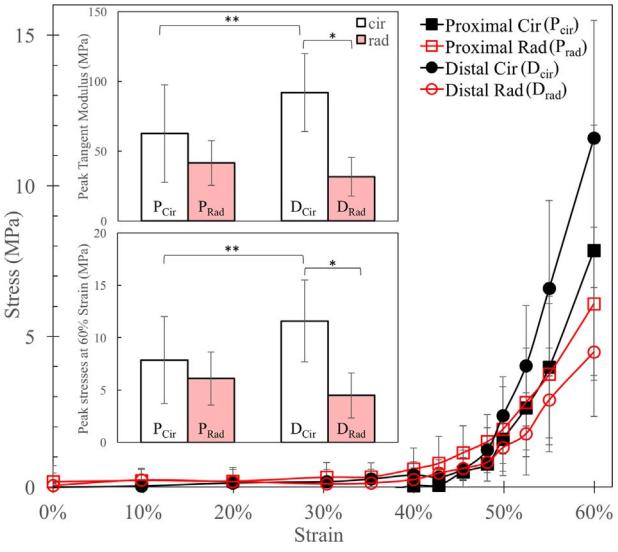

Fig. 2. Stress-strain curves for bovine saphenous venous valve tissue samples. For the first time, a nonlinear anisotropic mechanical property was observed in saphenous venous valves leaflets (mean saphenous vein valve leaflets by SEM; n = 36). Top inset - Inter-valvular variability in peak tangent moduli (in the 50–60% strain range); Bottom inset - peak stress values (at 60% strain) for saphenous venous valve tissues. Significant differences in peak tangent moduli and peak stress values at 60% strain between the circumferential and radial directions were found in the distal group, with anisotropic trends in the proximal and distal groups.

Based on statistical analysis, both groups had larger peak stresses at 60% strain in the circumferential direction (proximal group: 7.9 ± 4.2 MPa; distal group: 11.6 ± 3.9, $p = 0.04$) than those in the radial (proximal group: 6.1 ± 2.5 MPa; distal group: 4.5 ± 2.1, $p = 0.001$) (bottom inset of Fig. 2; Table 1), indicating that significant inter-valvular variability existed in the peak stresses at 60% strain in the circumferential direction. Recognizing that biaxial tensile testing retains axial coupling, it is noteworthy that peak stresses of 7.9 MPa (circumferential) and 11.6 MPa (radial) were measured at 60% strain, which are bracketed by uniaxial tensile strengths reported by Ackroyd et al. for human femoral vein valves, i.e., ~9 MPa (Ackroydd et al., 1985). Compared to jugular venous valve tissues (Huang and Lu, 2017), it appears that saphenous venous valve leaflets have higher peak tangent moduli and peak stresses at 60% strain. This could be due to the higher transvalvular pressure existing in saphenous veins and/or to thinner tissue thickness in the saphenous venous valve tissues (~30 μm), where the thickness of jugular venous valve tissue is ~50 μm. Detailed physiological factors are unclear and warrant further investigation. Moreover, previous study has shown that jugular venous valve tissues were stiffer than vein wall tissues (Kaul and Huang, 2017). Based on the biaxial testing results, despite the small dimensions of the saphenous venous valve samples, saphenous valves showed relatively larger peak tangent moduli and peak stresses at 60% strain (Table 1), which indicated that to design functional venous valves for CVI treatment, the prosthetic valve leaflets should possess relatively larger peak tangent moduli and peak stresses at 60% strain so as to serve as desired transplants.

During the dissection process, slight changes in the vein diameter were observed from the proximal to distal end along the vein. Also, as blood flows back to the heart from the distal to the proximal end, the valves from different locations on the vein are obviously exposed to different levels of hydrostatic pressure. These geometric and pressure conditions could also account for the differences between the mechanical properties of the proximal and distal leaflets. By comparing the results, distal tissues appeared to possess larger peak tangent modulus of elasticity in the circumferential direction as compared with proximal tissues (Fig. 2). As in the saphenous veins, blood flow comes from the distal end back to the proximal end, and the pressure level at the distal end should be significantly larger than that at the proximal end. Also, the blood in the leg flows against gravity, which requires the valves to be stronger so as to prevent potential reflux under either high blood pressure or effects from gravity.

The histological images of the tissues showed that collagen fibers in proximal saphenous vein samples did not exhibit a consistent orientation (Fig. 3a-c), indicating that there may be less directional dependency in their mechanical properties (Fig. 2). The collagen fibers in distal samples had a unique orientation (Fig. 3d-f), suggesting that the observed anisotropic mechanical properties in Fig. 2 could be related to the collagen fibers lining up well along the circumferential direction. In this case, the collagen fiber orientation might be one of the major factors which lead to the inter-valvular variability between leaflets

Table 1
Peak tangent moduli (in the 50–60% strain range) and peak stresses (at 60% strain) measured for venous valve leaflet tissues excised from bovine saphenous and jugular veins. Mean ± standard deviation (Huang and Lu, 2017).

Unit: MPa	SV Proximal		SV Distal		JV Proximal#		JV Distal#	
	Cir	Rad	Cir	Rad	Cir	Rad	Cir	Rad
Peak tangent modulus	62.7 ± 34.9	41.6 ± 16.0	92.1 ± 27.9	31.8 ± 13.9	26.9 ± 9.4	8.9 ± 5.6	29.7 ± 6.2	15.4 ± 9.5
Peak stress at 60% strain	7.9 ± 4.2	6.1 ± 2.5	11.6 ± 3.9	4.5 ± 2.1	6.3 ± 2.3	2.46 ± 1.3	6.12 ± 1.9	3.8 ± 2.7

from different longitudinal locations.

Eleven specimens from the proximal group and 11 from the distal group were collected for collagen assay. From these results, collagen concentration decreased from the proximal to the distal end (Fig. 4). The mean collagen concentration of proximal valves was 336,940 ± 92,360 μg/g wet weight, which was larger than that for the distal valves, which had 263,940 ± 68,000 μg/g wet weight. Nevertheless, no significance differences between these groups were observed. The lower concentration found in distal samples is attributed to the wet weight in the current study. Since distal samples have higher average wet weight, lower collagen concentration is obtained. Although distal samples have higher mechanical strength than that of proximal samples, previous studies have shown that elasticity moduli are correlated with collagen cross-link concentration, rather than collagen concentration in the leaflets (Huang et al., 2012, 2014; Huang and Lu, 2017; Huang and Huang, 2015). The acid-pepsin soluble collagen concentrations measured in this study for bovine saphenous venous valve leaflet tissues were comparable with those we measured previously for bovine jugular venous valves (Huang and Lu, 2017). In particular, for the proximal and distal jugular venous valve leaflets, we previously measured acid-pepsin soluble collagen concentrations of 301,000 ± 172,000 μg/g wet weight and 544,000 ± 234,000 μg/g wet weight, respectively (Huang and Lu, 2017). There are several caveats to consider when comparing the results reported herein for saphenous venous valve leaflets versus those for jugular venous valve leaflets in our previous study. By virtue of their significantly smaller thickness (~30 μm in saphenous venous valve leaflets vs ~50 μm in jugular venous valve leaflets), the wet weights of the saphenous venous valve leaflet samples utilized in the collagen assay (e.g., avg. 0.00078 g for proximal valve samples and avg. 0.001 g for distal valve samples) were lower than those of the bovine jugular venous valve (avg. 0.002 g for proximal valve samples) leaflet samples (Huang and Lu, 2017). Because the collagen concentration is normalized to the wet weight, weight measurement errors (e.g., due to water droplets, etc.) could potentially have been amplified. However another, and perhaps more important, consideration is that the collagen concentrations reported here for saphenous venous valve leaflet tissues and in our previous studies (Huang

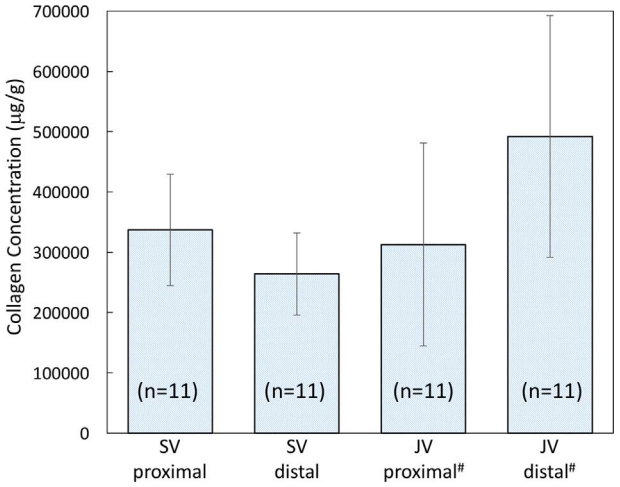

Fig. 4. Collagen concentrations measured following acid-pepsin extraction times of 120 h. A proximal to distal trend of decreased collagen concentration of saphenous venous valve leaflets was observed and is the reverse of the trend observed in jugular venous valve leaflets (Huang and Lu, 2017).

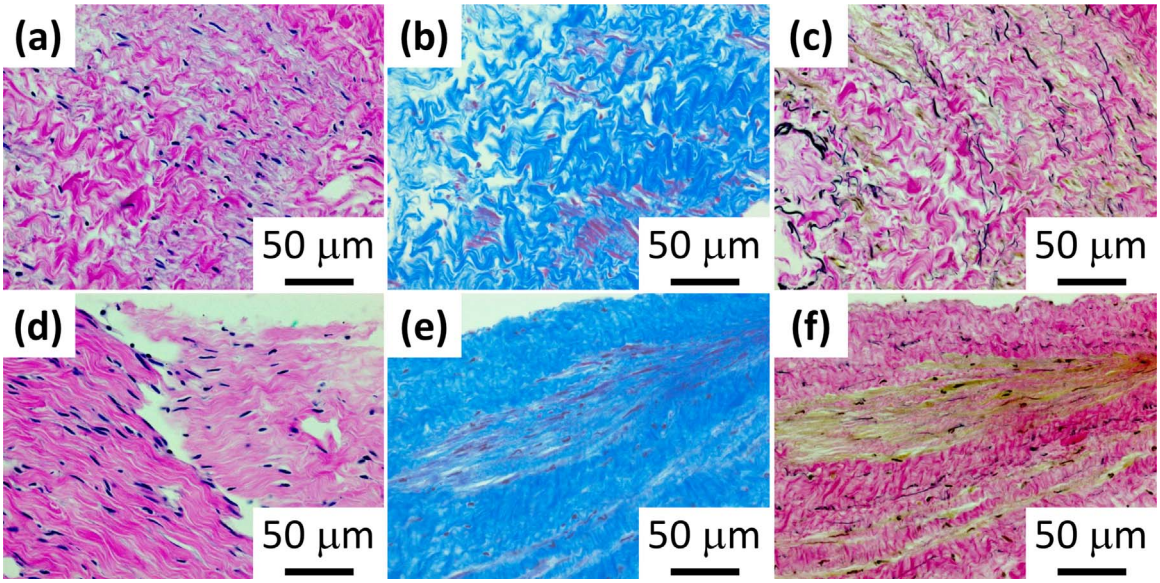

Fig. 3. Histological images of saphenous venous valve tissues (400x). (a)-(c) Proximal samples stained by H & E, Masson's Trichrome and VVG protocols, respectively. (d)-(f) Distal samples stained by H & E, Masson's Trichrome, and VVG protocols, respectively. Collagen fiber crimp (i.e., undulations) and circumferential alignment were observed in all distal samples, while collagen fiber alignment appeared qualitatively less pronounced in proximal venous valve leaflet sections. Importantly, elastic fibers were observed in both proximal and distal VVG stained sections (panels (c) and (f), respectively).

and Lu, 2017) on jugular venous valve leaflet tissues represent only the acid-pepsin soluble fraction of the total collagen. During the collagen assay experiments, only soluble collagen components were measured and analyzed. Insoluble collagen components such as hydroxyproline (HYP), elastin, and GAGs were not analyzed in this study. As those components should also contribute to the tissue properties, further chemical assay will be conducted in the future. Also, to better understand the structure of the components of the tissue, immuno-histochemical samples will be prepared for confocal microscopy.

As a pilot study in the field, certain limitations existed in this study. Based on our observation during the dissection process, one leaflet is generally smaller than the other one, and the smaller leaflet is with length L = 3–5 mm and leaflet height h = 0.5–1 mm. Such leaflet is too small to be mounted by our customized clamping system (Fig. 1c) and is excluded from our experiments. Due to the lack of physiological information regarding native loading conditions for venous valve tissues, the mechanical testing was conducted under a strain rate of 1%/s to 60% strain, as prior to failure. However, taking the viscoelasticity of the valve tissues into account, the loading conditions and rate would affect the properties of the tissues. In this case, further information from physiological studies will be needed to adjust the testing protocol. As in the case of shear conditions, pure shear provides important information in tissue mechanics; we are currently in the process of establishing proper protocols to measure shear stress and strains for the venous tissues. Moreover, bovine saphenous veins were used in lieu of human tissues due to their availability and use in the fabrication of prosthetic valves. It is expected that differences will likely be observed between bovine and human venous valve leaflet tissue mechanical properties.

4. Conclusions

For the first time, anisotropic nonlinear behavior in bovine saphenous venous valve leaflet tissues was measured via equibiaxial mechanical loading. The peak tangent moduli of the tissues and the peak stresses at 60% strain were obtained. Valve leaflets from proximal saphenous veins did not show clear anisotropic mechanical properties, while leaflets from distal saphenous veins showed significant anisotropic properties, with the peak tangent modulus of elasticity in the circumferential direction being three times as large as that in the radial direction. It is also observed that both peak tangent moduli and peak stresses at 60% strain are higher for distal samples than those for proximal samples. It is suggested that larger hydrostatic pressure at the distal end of veins might be one of the root causes for stiffer distal valvular tissues, allowing them to close completely during venous return. Based on collagen assay results, a decreasing trend in collagen concentration was observed from proximal to distal valve tissues. These results indicated that the components in the valve tissues may be location-dependent. This study has the potential to transform not only the basic understanding of physiological structure-function correlations in venous valves, but also to serve as a springboard for innovative, informed approaches to the treatment of CVI by way of providing, for the first time, a detailed biaxial mechanical characterization of saphenous venous valve leaflets. The study will culminate in synergistic activities between experimental and clinical efforts aimed at a breakthrough in clinical treatments for damaged venous valve tissue with a corresponding improvement in quality of life.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

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