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Strain effects on collagen proteolysis in heart valve tissues

Kaitlyn Barbour[1] · **Hsiao-Ying Shadow Huang**[1]

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Abstract Collagen is at the heart of any and all questions concerning semilunar valvular leaflet composition, structure, and function. Whether during development, physiological homeostasis, or pathological degeneration, it is the structural-mechanical state of the heart valve leaflet collagen network that ultimately confers valvular function, and the difference between health and disease. In the current study, the effects of physiologically relevant strain states on collagen catabolism are investigated in porcine aortic and pulmonary valve leaflets. Application of bacterial collagenase to the tissues which acts to simulate collagen degradation by endogenous matrix metalloproteinases, biaxial stress relaxation, and histology are all used to serve as measures of functional and compositional collagen catabolism. Current stress-relaxation results are used in conjunction with previous equibiaxial testing to confirm that a mechanism exists to prevent collagen catabolism when stretched at physiologically relevant strain states. Collectively, these in vitro results indicate that biaxial strain states are capable of impacting the susceptibility of valvular collagens to catabolism, and that at physiological strain states, a protective mechanism exists to effectively block collagen catabolism. The results of the study will be broadly applicable to clarify the roles of tissue microarchitecture and load transmission in a variety of other developmental, homeostatic, or pathogenic tissue processes such as tumor growth, embryogenesis, thrombi formation, and atherogenesis.

Keywords Physiological strains · Biomechanical testing or analysis · Tissue biomechanics · Stress relaxation

1 Introduction

In the United States, valvular heart disease accounts for approximately 25,000 deaths each year, with roughly 250,000 people presently suffering from heart valve diseases. With an

✉ H.-Y.S. Huang
hshuang@ncsu.edu

[1] Mechanical and Aerospace Engineering Department, North Carolina State University, R3158 Engineering Building 3, 911 Oval Drive, Campus Box 7910, Raleigh, NC 27695, USA

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increasing population, the number of patients with severe heart valve disease continues to rise (Benjamin et al. 2017; NIH 2016; Kheradvar et al. 2015). Heart valve diseases are caused by the disruption of homeostatic mechanisms within the valve and leaflets that alter the microstructural properties of the tissue. Many factors such as calcification and mechanical stress on the valve and leaflets can bring on valvular diseases (Thubrikar et al. 1980, 1982, 1983, 1986; Schoen and Levy 2005; Schoen 2016; Yip et al. 2009; Yip and Simmons 2011). Healthy leaflets imply homeostasis, with subpopulations of valvular interstitial cells (VICs) catabolizing damaged collagen and the same or other VICs mediating *de novo* collagen synthesis (Merryman et al. 2007; Rabkin-Aikawa et al. 2004; van der Kamp and Nauta 1979). By contrast, disruption, depletion, or excess accretions of collagens are hallmarks of various valvular diseases (Rabkin et al. 2001; Sacks and Schoen 2002; Cole et al. 1984; Banerjee et al. 2014; Lacerda et al. 2012), likely manifesting as both causes and effects of disrupted mechanotransduction across the macro to micro length scales. These two sides of the remodeling coin beg the question: what are the signals that instruct VICs to turnover collagen? Non-physiological stresses and strains are capable of eliciting VIC secretion of collagenolytic matrix metalloproteinases (MMPs) (Balachandran et al. 2009; Fondard et al. 2005), such as in the high stress vicinity of calcific nodules (Perrotta et al. 2014), and likely mediated through changes in VIC deformation (Huang et al. 2007, 2014; Carruthers et al. 2012; Lewinsohn et al. 2011; Anssari-Benam et al. 2012). However, in what way would VIC-secreted MMPs differentiate damaged collagen from functional, structurally load-bearing fibers? The answer may lie in load bearing itself. In 1977, Chor Huang and Ioannis V. Yannas demonstrated that the susceptibility of bovine tendon-derived collagen to enzymatic cleavage can depend on the strain state of the fiber (Huang and Yannas 1977). Studies by Nabeshima et al. on reconstituted collagen (Nabeshima et al. 1996), and more recently by the laboratory of Jeffrey Ruberti (Bhole et al. 2009; Camp et al. 2011; Robitaille et al. 2011; Flynn et al. 2010, 2013; Zareian et al. 2010; Ruberti and Hallab 2005; Chang et al. 2012), support the hypothesis that collagen exhibits "mechanochemical switches." Collagen fiber strain may effectively block proteolytic sites through triple-helix structural rearrangement (Flynn et al. 2013; Camp et al. 2011), thereby conferring resistance to degradation, leaving unloaded fibers selectively susceptible to collagenases and MMPs (Flynn et al. 2010; Ghazanfari et al. 2016). Conceptually, this paradigm has broader implications for developmental tissue growth, physiological load adaptation, and pathological degeneration (Bhole et al. 2009; Ruberti and Hallab 2005). Nevertheless, it remains unclear how such strain-dependent phenomena may manifest in heart valve leaflets, which exhibit non-orthogonal collagen fiber orientation distributions and complex loading states.

Unlike tendons, heart valve leaflets exhibit relatively broad planar distributions of collagen fiber angular orientation (Sacks et al. 1997), and both circumferential and radial physiologic loading components, a stress-strain state amenable to simulation by biaxial mechanical testers (Huang et al. 2012). Together, these structural and physiological loading properties confer slack and induce fiber rotations, factors which collectively confound direct relationships between macro-scale, tissue-level strain and the state of strain within individual collagen fibers (Ellsmere et al. 1999; Aldous et al. 2009). Ruberti et al. have leveraged the orthogonal collagen fiber orientations of corneal tissue to demonstrate selective degradation of intrinsic "control fibers" perpendicular to the loading axis (Robitaille et al. 2011; Ruberti and Hallab 2005; Zareian et al. 2010). To date, however, the closest evidence for collagen fiber strain-state-dependent catabolism that is directly relevant to heart valves comes from studies of bovine pericardium (Ellsmere et al. 1999), a tissue routinely utilized in the fabrication of chemically cross-linked bioprosthetic heart valves.

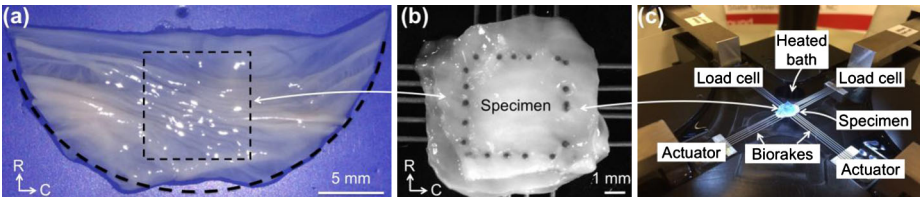

Fig. 1 (**a**) Leaflet sample dissected from aortic valve, cut along the valve wall (dashed line). (**b**) Leaflet samples were cut 7 mm × 7 mm and placed on biorakes with tines pointing upward. (**c**) Biotester 5000 with labeled actuators and load cells

Previously, stress-relaxation tests similar to those of Huang and Yannas (1977) were conducted on aortic and pulmonary valve leaflet tissues to investigate the effects of equibiaxial strain state on collagen catabolism. Similar to recent work by Rodriguez et al. (2014), bacterial collagenase was utilized to simulate endogenous MMPs, and equibiaxial stress relaxation and biochemical collagen concentration served as functional and compositional measures of collagen catabolism, respectively. With increased collagenase concentrations there was a significantly lower magnitude of collagen content, and with increased strain imposed on the leaflet specimens an accelerated normalized stress-relaxation rate was observed. Collectively, these *in vitro* results indicate that biaxial strain state is capable of impacting the susceptibility of valvular collagens to catabolism, providing the basis for further investigation of how such phenomena may manifest at different strain magnitudes, the foundation for this study (Huang and Huang 2015).

The objective of this study was to determine if *physiologically relevant* biaxial strain states are capable of influencing the susceptibility of collagen to catabolism and if these strain states induce a protective mechanism that protects collagen from degradation. We hypothesize that at physiological strain states, a protective mechanism exists to prevent collagen catabolism, resulting in lower magnitudes of stress relaxation and little change in the collagen fiber microstructure. To the best of the authors' knowledge, the study was the first to investigate such phenomena in the context of valvular tissues.

2 Methods

2.1 Tissue procurement and collagenase preparation

Thirty-six porcine hearts were obtained from the Nahunta Pork Center (Pikeville, NC) and aortic and pulmonary valves were excised. Each leaflet was dissected from the aortic valves (AVs) or pulmonary valves (PVs) by cutting against the wall of the artery (Fig. 1a) and stored refrigerated (4 °C) in Hank's Balanced Salt Solution (HBSS; Lonza, Walkersville, MD) without protease inhibitors. Mechanical testing were performed no later than 24 hours after dissection, and 7 mm × 7 mm samples were cut from the center of the leaflet (Fig. 1b), ensuring the sample axes corresponded to the circumferential (C) and radial (R) directions of the leaflet. Samples were placed on biaxial tester rakes (BioTester 5000; CellScale, Waterloo, ON, Canada) and lowered into a 37 °C HBSS bath (Fig. 1c). Detailed description of the biaxial tester for heart valve tissues were descried in Huang et al. (2012).

Bacterial collagenase solutions were prepared for use in testing to simulate the effects of endogenous MMPs on the leaflet tissue. Collagenase solutions (0.2 and 0.5 mg/mL) were prepared by dissolving collagenase type 2 derived from *Clostridium histolyticum* (Worthing-

ton Biochemical Corp.; Lakewood Township, NJ) in HBSS. These solution concentrations were chosen based on our previous study (Huang and Huang 2015) to allow us to compare data. To prepare the solution, 25 mg or 62.5 mg of collagenase powder (for 0.2 mg/mL and 0.5 mg/mL solutions, respectively) were measured using an analytical balance and dissolved in 125 ml of HBSS. Bacterial collagenase was intended to degrade intact collagen type I fibers to mimic the mechanism of proteolysis by MMPs (Ellsmere et al. 1999). The collagenase type 2 was stored refrigerated at 4 °C until dissolved in solution in preparation for mechanical testing and placed in a heated water bath (37 °C) until needed.

2.2 Biaxial stress relaxation testing

Unlike tendons, heart valve leaflets exhibit relatively wide planar distributions of collagen fiber orientation as well as circumferential and radial physiologic loading components that create a complex anisotropic tissue. Understanding how strain states affect the susceptibility of collagen to catabolism is key to determining if a mechanism exists that protects or accelerates the proteolysis of collagen fibers in heart valve leaflets. To determine if there was some sort of protective mechanism that prevented collagen degradation when stretched to physiological strain levels, biaxial stress-relaxation tests were performed. For detailed biaxial stress-relaxation tests, please refer to our previous study on effects of collagenase concentration and equibiaxial strain state (Huang and Huang 2015).

Mounted samples on the BioTester were subjected to a pre-conditioning of 2% strain in both the circumferential (C) and radial (R) directions. Ten cycles, each 5 s of stretch and 5 s of recovery, were applied to eliminate tissue tearing during experimental testing. The preconditioning cycles were followed by a 30 s rest period. The physiological strains for AV and PV at both circumferential and radial directions were determined by an equibiaxial force-controlled mechanical testing, as described in Sacks and Yoganathan (2007). For experimental testing in the current study, the AV was stretched to C:R = 20%:70% (C:R ratio = 1:3.5) and the PV was stretched to C:R = 20%:90% (C:R ratio = 1:4.5) (Sacks and Yoganathan 2007). These strain levels were chosen based on physiological transvalvular pressures applied when the valve is fully closed to prevent regurgitation of blood back into the ventricles (Sacks and Yoganathan 2007). Stress-relaxation testing allowed the tissue to undergo the strains it would be exposed to physiologically when the valve was completely closed. Strain levels were increased in four incremental steps until the desired strain level was reached. AV was stretched to C:R = 5%:17.5% for four steps and PV was stretched to C:R = 5%:22.5% for four steps. It took a total of 35 s to reach the desired strain levels.

The time at which the desired strain levels were reached was designated time zero ($t = 0$). Followed by the protocol of stress-relaxation testing established by Sacks research group (Stella et al. 2007), as well as our previous study on equi-biaxial stress relaxation of heart valve leaflets (Huang and Huang 2015), samples were held at those strain levels for 10,000 s. At 3,000 s, the HBSS was removed from the bath using a serological pipet and pipet aid (Powerpette; VWR, Radnor, PA). For each experimental condition, the bath was refilled with either 0.2 mg/mL or 0.5 mg/mL collagenase solution previously prepared and heated to 37 °C. For control samples, the bath was refilled with 37 °C HBSS. Based on our previous study, it was observed that 10,000 s was adequate for collagenase to diffuse through the tissues during the stress-relaxation experiments. For each experimental condition at varying strain levels and collagenase concentrations, six samples for each collagenases concentrations (including control groups) were tested (from a total of 18 hearts). Load values registered during stress relaxation were converted to nominal stress based on sample dimensions, and the associated normalized stresses were calculated by dividing the stress at each time point by the initial stress at $t = 0$.

Table 1 α and β values for stresses along the circumferential directions of the AV and PV tissues. The parameters were determined by fitting the experimental data for both physiological strain levels and previous equibiaxial strain levels (Huang and Huang 2015) to Eq. (1): $\sigma_m(t) = \sigma_p - \alpha t^{\beta}$

AV		Control	0.2 mg/ml	0.5 mg/ml	PV		Control	0.2 mg/ml	0.5 mg/ml
20%:70% (1:3.5)	α	22.083	7.118	4.729	20%:90% (1:4.5)	α	33.329	2.592	1.771
	β	0.092	0.291	0.361		β	0.103	0.390	0.529
25%:87.5% (1:3.5)	α	31.192	2.681	3.345	25%:112.5% (1:4.5)	α	31.415	2.686	1.626
	β	0.1083	0.3945	0.4334		β	0.072	0.414	0.571
37.5%:37.5% (1:1)	α	184.636	7.180	10.50	37.5%:37.5% (1:1)	α	118.125	1.538	2.716
	β	0.0714	0.4660	0.5027		β	0.053	0.569	0.508
50%:50% (1:1)	α	210.731	6.540	5.292	50%:50% (1:1)	α	172.611	5.428	3.194
	β	0.0811	0.5062	0.5372		β	0.078	0.509	0.579

Additional 18 hearts were also precured to determine if this protective mechanism is present only at the tested strain level (C:R = 20%:70% for AV and C:R = 20%:90% for PV) or if it holds true at any other strain levels at the same ratios C:R = 1:3.5 for AV and C:R = 1:4.5 for PV. Therefore, a second stress-relaxation test for AV ($n = 6$ for each collagenase concentration) was performed at 25%:87.5% strain (also at a 1:3.5 ratio), and a second stress-relaxation test for PV ($n = 6$ for each collagenase concentration) was also performed at 25%:112.5% strain (also at a 1:4.5 ratio).

2.3 Modeling

Similar to Wyatt et al. (2009), a one-dimensional three-parameter power function of time was used to describe the stress-relaxation response $\sigma_m(t)$ of the tissue after collagenase solution was introduced, given by

$$\sigma_m(t) = \sigma_p - \alpha t^{\beta}, \tag{1}$$

where σ_p is the peak stress at the end of mechanical elongation (or initial stretch), t is time, and α and β are relaxation constants. The relaxation constants were determined for each experimental condition from the least-squares best-fit of Eq. (1) to the experimental data (Table 1) using MATLAB (MathWorks, Natick, MA).

2.4 Histology

Histology was implemented to see what the tissue and its extracellular matrix (ECM) components looked like at the end of mechanical testing. Upon completion of biaxial testing, HBSS was removed from the BioTester bath with a powered pipet controller and 10% neutral buffered formalin were added back into the bath to fix the tissues. AV and PV samples for each experimental condition were then removed from the rakes and put in a prefilled container of 10% neutral buffered formalin (VWR, Radnor, PA) and refrigerated (4 °C) for 24 hours. Samples were prepared at the Histology Lab in the College of Veterinary Medicine at the North Carolina State University, paraffin embedded, sectioned, stained with Masson's Trichrome (for collagen) or Verhoeff-Van Gieson (for elastin) and put on histological slides to be digitized as micrographs. Slides were micrographed using an inverted microscope (VWR VistaVision™) at 400× magnification.

3 Results and discussion

3.1 Biaxial stress relaxation

To determine if there was some sort of protective mechanism that prevented collagen degradation when stretched to physiological strain levels, stress-relaxation tests were performed. These tests were compared to previously completed tests at equibiaxial strain levels, strains that are not natural to the aortic or pulmonary valves (Huang and Huang 2015). For the aortic valve, leaflets were stretched to 20% strain in the circumferential direction and 70% in the radial direction, as shown in solid lines. This was compared to our previously published data for an equibiaxial test at 37.5% in both directions (presented in dashed lines from Huang and Huang 2015) (Fig. 2a). Stress measurements were normalized to allow for comparison of each sample and experimental condition. The equibiaxial tests showed a significant drop in stress after the collagenase solutions were introduced at 3,000 s, while the physiological strain level tests (i.e., AV was stretched to C:R = 20%:70% and the PV stretched to C:R = 20%:90%) had a much lower stress drop, even after collagenase was introduced to degrade the collagen at 3,000 s. This indicates that there exists some sort of protective mechanism that prevents degradation of collagen, allowing the leaflet samples to remain in good condition mechanically with little stress relaxation compared to non-physiological applied strains.

To determine if this protective mechanism is present only at the tested physiological strain level or if it holds true at another strain levels at the same ratio (C:R = 1:3.5), a second stress-relaxation test was performed at 25%:87.5% strain (also at a 1:3.5 ratio). The normalized stress was compared to 20%:70% strain (Fig. 2b), and presented little difference in stress relaxation with collagenase (0.2 mg/mL and 0.5 mg/mL), suggesting the protective mechanism may be present at any strain level with a ratio of 1:3.5. Please note that error bars were taken away for clear presentation. Alternatively, please see Fig. 6a for the same data where error bars were all included.

Similar tests were completed for the pulmonary valve. Leaflets were stretched to 20% strain in the circumferential direction and 90% in the radial direction. This was compared to our previously published data for an equibiaxial test at 37.5% in both directions (presented in dashed lines Huang and Huang 2015) (Fig. 3a). Again, the equibiaxial tests showed a significant drop in stress after the collagenase solutions were introduced, while the physiological strain level tests had a much lower stress drop. There was a clear indication that some sort of protective mechanism exists to prevent the degradation of collagen when stretched to physiologically relevant strain levels. To determine if this protective mechanism was present at different strain levels with the same ratio (20%:90% = 1:4.5), a second stress-relaxation test was performed at 25%:112.5% strain (C:R = 1:4.5). The normalized stress was compared to 20%:90% strain, and again presented little difference in stress relaxation (Fig. 3b), suggesting the protective mechanism may be present at any strain level with a ratio of 1:4.5. Please note that error bars were taken away for clear presentation. Alternatively, please see Fig. 6b for the same data where error bars were all included.

Anssari-Benam et al. studied porcine AV leaflets without collagenase subjected to uniaxial stress-relaxation tests in both the circumferential and radial directions (Anssari-Benam et al. 2011, 2012). Unlike cyclic stretching, stress-relaxation tests give insight into the time-dependent behavior of the matrix components of the AV leaflet. Their data suggested that the time-dependent behavior was highly reliant upon both the direction (circumferential or radial) and magnitude of the applied strain. As the applied strain increased, the amount of relaxation decreased and the time it took to get to a relaxed state increased, suggesting some hindrance to the relaxation process. Again, this study points to

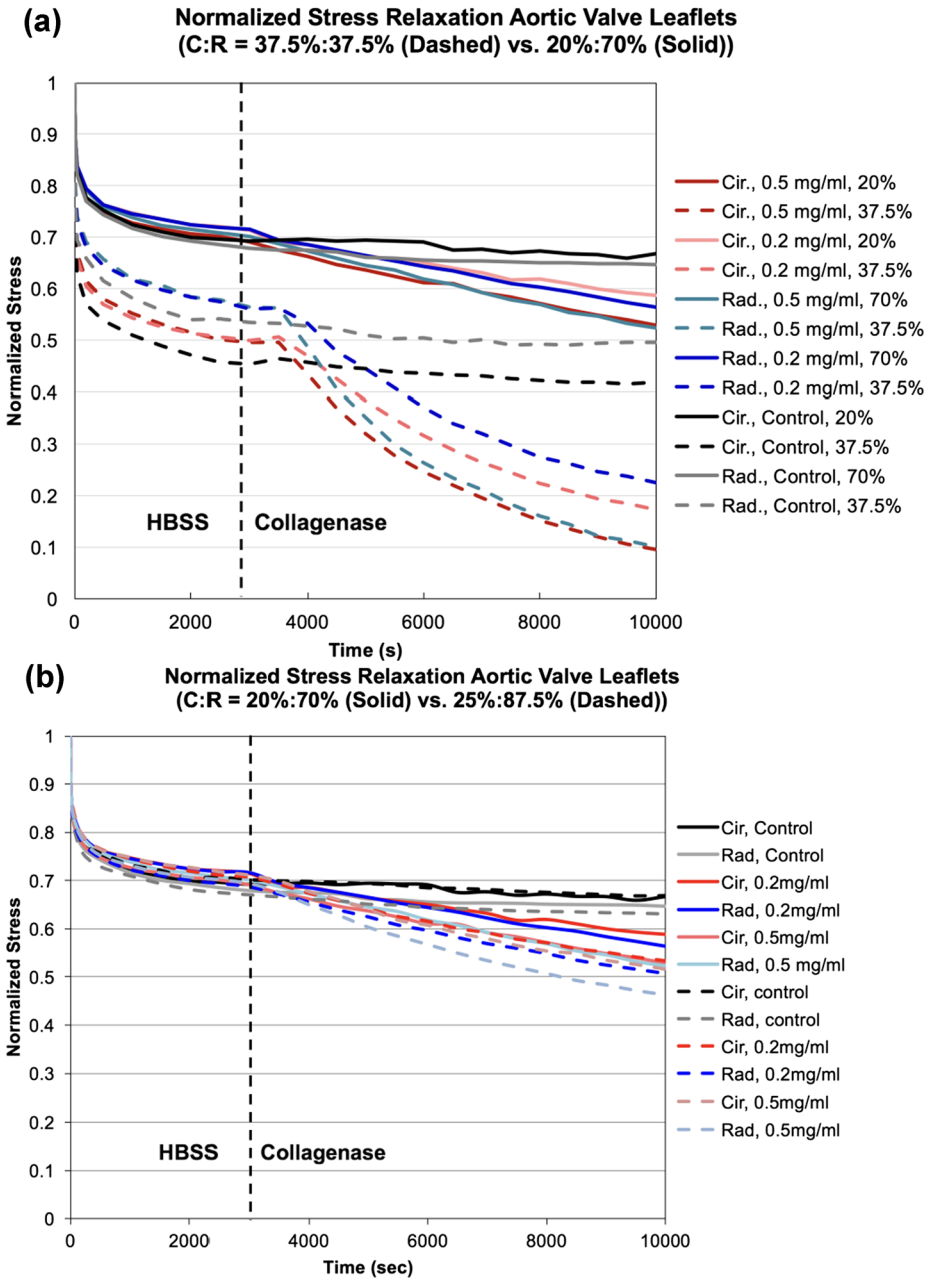

Fig. 2 (**a**) Normalized stress of the aortic valve leaflets at C:R = 37.5%:37.5% equibiaxial strain (dashed lines) (Huang and Huang 2015) compared to C:R = 20%:70% strain (solid lines). (**b**) Normalized stress of the aortic valve leaflets C:R = 20%:70% strain (1 to 3.5 ratio; solid lines) compared to C:R = 25%:87.5% strain (other strain levels at the same ratio [i.e., 1 to 3.5]; dashed lines). For the collagenase-treated groups, HBSS was replaced with 0.2 mg/mL or 0.5 mg/mL collagenase solution after 3,000 s; for the control group (i.e., 0 mg/mL), HBSS was not replaced (Color figure online)

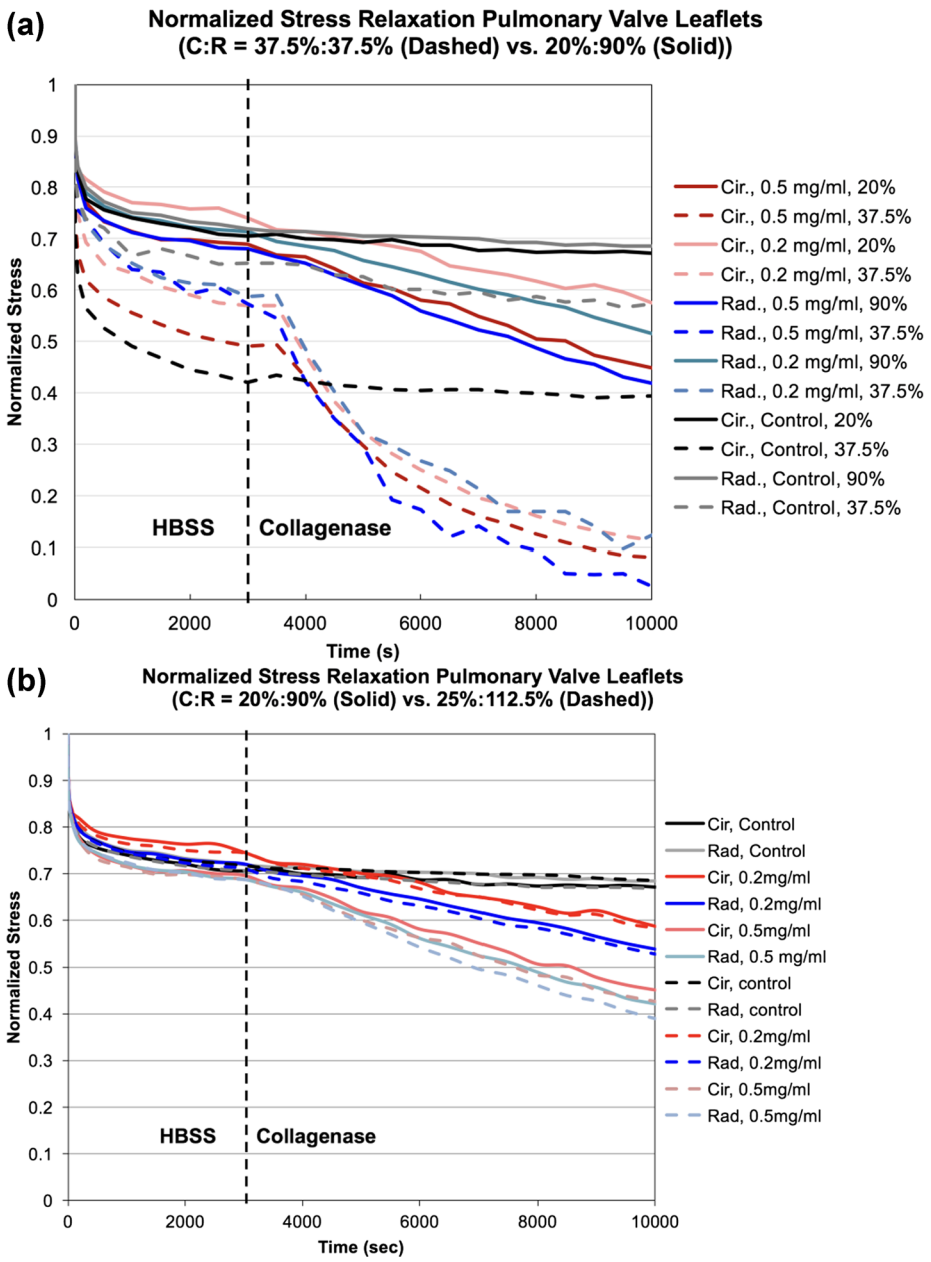

Fig. 3 (**a**) Normalized stress of the pulmonary valve leaflets at C:R = 37.5%:37.5% equibiaxial strain (dashed lines) (Huang and Huang 2015) compared to C:R = 20%:90% strain (solid lines). (**b**) Normalized stress of the aortic valve leaflets C:R = 20%:90% strain (solid lines) compared to C:R = 25%:112.5% strain (i.e., 1 to 4.5; dashed lines). For the collagenase-treated groups, HBSS was replaced with 0.2 mg/mL or 0.5 mg/mL collagenase solution after 3,000 s; for the control group (i.e., 0 mg/mL), HBSS was not replaced (Color figure online)

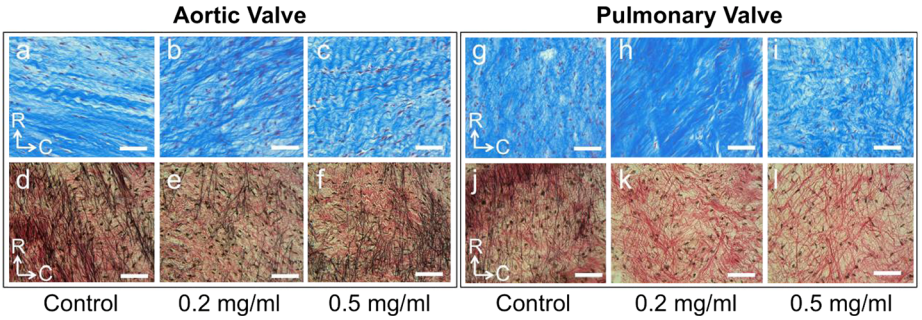

Fig. 4 AV at 20%:70% strain for Trichrome stain at 400× magnification for (**a**) control, (**b**) 0.2 mg/mL collagenase concentration, and (**c**) 0.5 mg/mL collagenase concentration, and for VVG stain at (**d**) control, (**e**) 0.2 mg/mL collagenase concentration, and (**f**) 0.5 mg/mL collagenase concentration. PV at 20%:90% strain for Trichrome stain at 400× magnification for (**g**) control, (**h**) 0.2 mg/mL collagenase concentration, and (**i**) 0.5 mg/mL collagenase concentration, and for VVG stain at (**j**) control, (**k**) 0.2 mg/mL collagenase concentration, and (**l**) 0.5 mg/mL collagenase concentration. Scale bar = 50 μm

the idea that a mechanism exists to protect collagen fibers from degradation at a certain strain state (Anssari-Benam et al. 2011, 2012; Lewinsohn et al. 2011; Huang et al. 2014; Huang and Huang 2015).

3.2 Statistics

Statistical analyses were conducted using GraphPad Prism Software involving one factor (e.g., normalized stress at $t = 10{,}000$ s) and was conducted by one-way ANOVA, with Tukey's post-hoc test for significance (p-value < 0.05) of multiple comparisons. Multiple factors (e.g., strain magnitude and collagenase concentration) were tested by two-way ANOVA.

3.3 Histology

In general, histology was vital to provide a visual representation of the state of fibers in experimental samples. Masson's Trichrome stain was used to visualize collagen fibers at the completion of mechanical testing and Verhoeff-Van Gieson (VVG) stain was used to see elastin fibers. Collagen was predominantly aligned in the circumferential direction, while elastin was predominantly aligned in the radial direction in the leaflets. In its native state, collagen fibers have a natural crimp, loose crimp at $\sim 3\%$ axial strain, and are fully extended only when stretched to high strain levels. Based on the trichrome stained samples, it was clear that the collagen fibers had remained in a crimped or wavy state even when fully stretched to physiological strain levels (Fig. 4). Even at increased concentrations of collagenase solutions, the crimping still existed in both the AV and PV samples. There was no indication of degradation of the collagen fibers, again indicating that there might exist a protective mechanism preventing the cleavage of collagen fibers at physiological strain levels.

Elastin ran predominantly in the radial direction as indicated by the VVG stained samples. In the AV samples (Fig. 4a–f), the elastin seemed to degrade or fragment with increasing collagenase concentration. This could likely be explained by the fact that the radial direction of the leaflet was stretched to higher strain levels than the circumferential, and in combination with the collagenase the elastin was not able to withstand the prolonged stretching (almost three hours) that was imposed upon it. In its native condition, the elastin

would have only been stretched long enough for the valve to close, and would then contract to open the leaflets to allow for blood flow. The PV samples had very little, if any, recognizable elastin.

3.4 Modeling

The relaxation constants α and β (Table 1) were determined by fitting Eq. (1) to the experimental data. The predicted stress-relaxation response was modeled for each experimental condition as shown in Fig. 5. In Fig. 5, our previously published data for an equibiaxial test (37.5% and 50%) (Huang and Huang 2015) were used to compare the effects of physiological strain states on proteolyzed leaflets. For the normalized circumferential stress of the AV, the model indicated a significant amount of stress relaxation for the equibiaxial strain levels (indicated in green and purple), compared to a much smaller stress drop for physiological strain levels (indicated in red and blue) (Fig. 5a). For the radial stress of the AV, there was a less clear pattern in the stress-relaxation model (not shown). This could likely be explained by the fact that most collagen was aligned in the circumferential direction and therefore the stress drop in the radial direction was dependent on the mechanical strength and ability of elastin fibers to hold the radial load, rather than those of collagen.

For the normalized circumferential stress of the PV samples, the model again indicated a significant amount of stress relaxation for the equibiaxial strain levels (indicated in green and purple), compared to a much smaller stress drop for physiological strain levels at low collagenase concentrations (indicated in red and blue) (Fig. 5b). At the highest tested collagenase concentration (0.5 mg/mL; indicated by short dashed lines), there was a larger stress-relaxation response, suggesting degradation of collagen fibers. Procine PV leaflets were known to be much thinner ($\sim$ 0.3 mm) than ones in AV ($\sim$ 0.6 mm) and therefore it is possible that there exists some threshold collagenase concentration that when reached, is able to degrade the collagen fibers regardless of strain level. Because of the thickness difference, the threshold may be lower for PV leaflets, which could explain why this result was not seen for AV samples but was present in PV samples (Fig. 5). For the radial stress of the PV samples, there was again a less clear pattern in the stress-relaxation models (not shown). This result could likely be a combination of the fact that less anisotropy was observed in the PV samples to begin with (Fig. 4g) and that the collagenase might begin to degrade the collagen fibers at some threshold concentration between 0.2 mg/mL and 0.5 mg/mL (Fig. 4h–i).

There were several study limitations in the current study. To determine if this protective mechanism is present only at the tested strain level (C:R = 20%:70% for AV and C:R = 20%:90% for PV) or if it holds true at any other strain levels at the same ratios C:R = 1:3.5 for AV and C:R = 1:4.5 for PV, several stress-relaxation tests for AV (C:R = 10%:35%, C:R = 15%:52.5%; a ratio of 1:3.5) and PV (C:R = 10%:45%, C:R = 15%:67.5%; a ratio of 1:4.5) were performed. However, we were unable to obtain any result due to following reasons: First, it was noted that AV and PV leaflets would stretch against the actuator if the given strain state was less than 20% along the circumferential direction. These strain levels (20% along the circumferential direction for both AV and PV leaflets) were chosen based on transvalvular pressures applied when the valve is fully closed to prevent regurgitation of blood back into the ventricles (Sacks and Yoganathan 2007). In addition, several other stress-relaxation test for AV (C:R = 25%:87.5%, C:R = 30%:105%; a ratio of 1:3.5) and PV (C:R = 25%:112.5%, C:R = 30%:135%; a ratio of 1:4.5) were performed. Leaflet samples started tearing off along the radial direction during the 10,000 second stress-relaxation testing, and it is due to the fact that such large strain levels along the radial direction for both

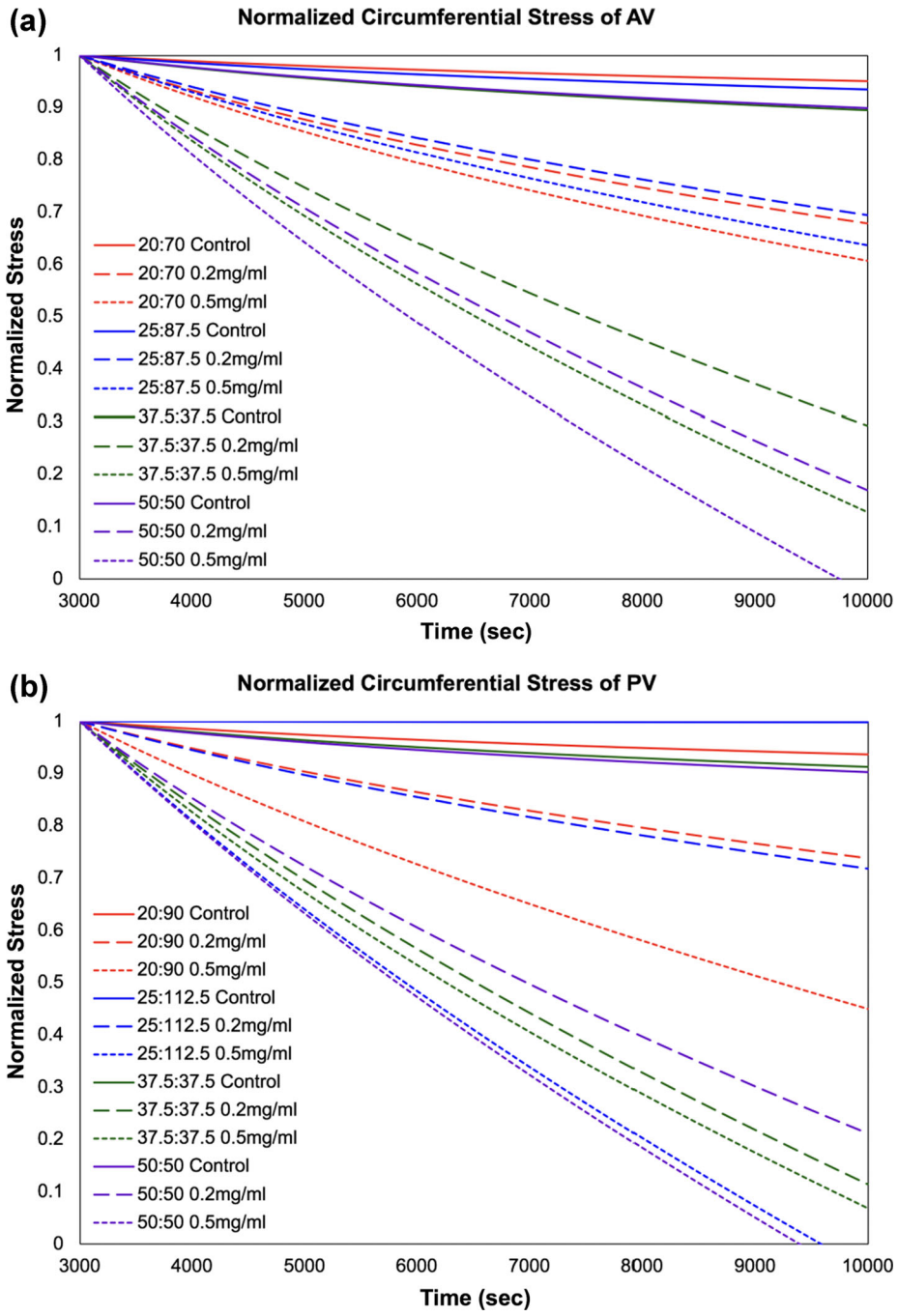

Fig. 5 For the normalized circumferential stress of the (**a**) AV and (**b**) PV, the model $\sigma_m(t) = \sigma_p - \alpha t^{\beta}$ showed a significant amount of stress relaxation for the equibiaxial strain levels (indicated in green and purple), compared to a much smaller stress drop for physiological strain levels (indicated in red and blue). α and β values are from Table 1 (Color figure online)

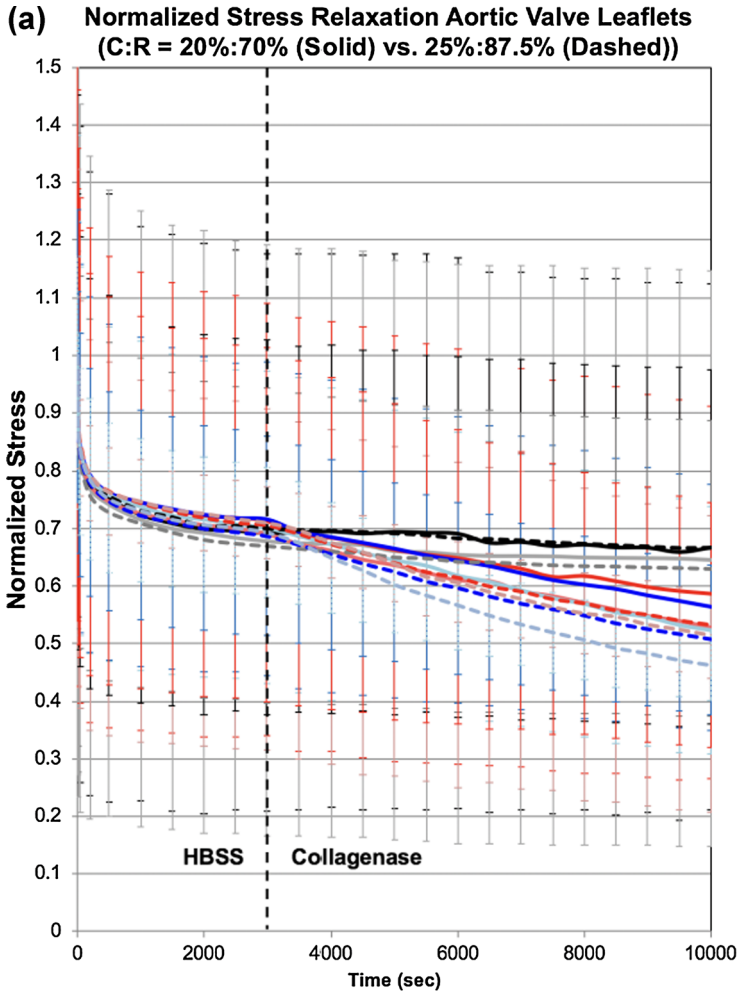

Fig. 6 (**a**) Normalized stress of the aortic valve leaflets C:R = 20%:70% strain (1 to 3.5 ratio; solid lines) compared to C:R = 25%:87.5% strain (other strain levels at the same ratio [i.e., 1 to 3.5]; dashed lines). For the collagenase-treated groups, HBSS was replaced with 0.2 mg/mL or 0.5 mg/mL collagenase solution after 3,000 s; for the control group (i.e., 0 mg/mL), HBSS was not replaced. (**b**) Normalized stress of the aortic valve leaflets C:R = 20%:90% strain (solid lines) compared to C:R = 25%:112.5% strain (i.e., 1 to 4.5; dashed lines). For the collagenase-treated groups, HBSS was replaced with 0.2 mg/mL or 0.5 mg/mL collagenase solution after 3,000 s; for the control group (i.e., 0 mg/mL), HBSS was not replaced (Color figure online)

AV and PV leaflets were above the stretch thresholds that leaflets could be subjected to. In addition, similar to the work by Rodriguez et al. (2014), in this study, bacterial collagenase at two different concentrations (0.2 mg/mL and 0.5 mg/mL) were utilized to simulate the effects of endogenous MMPs involved in physiologic valvular collagen remodeling. However, it was our study limitation to quantitatively relate these concentrations to MMP ranges. As for the further study, the Movat pentachrome staining method could be used to reveal more details to differentiate nuclei, elastin, GAGs, and collagen. Alternatively, Picrosirius Red-stained could be used for birefringence microscopy to better-visualize the directional

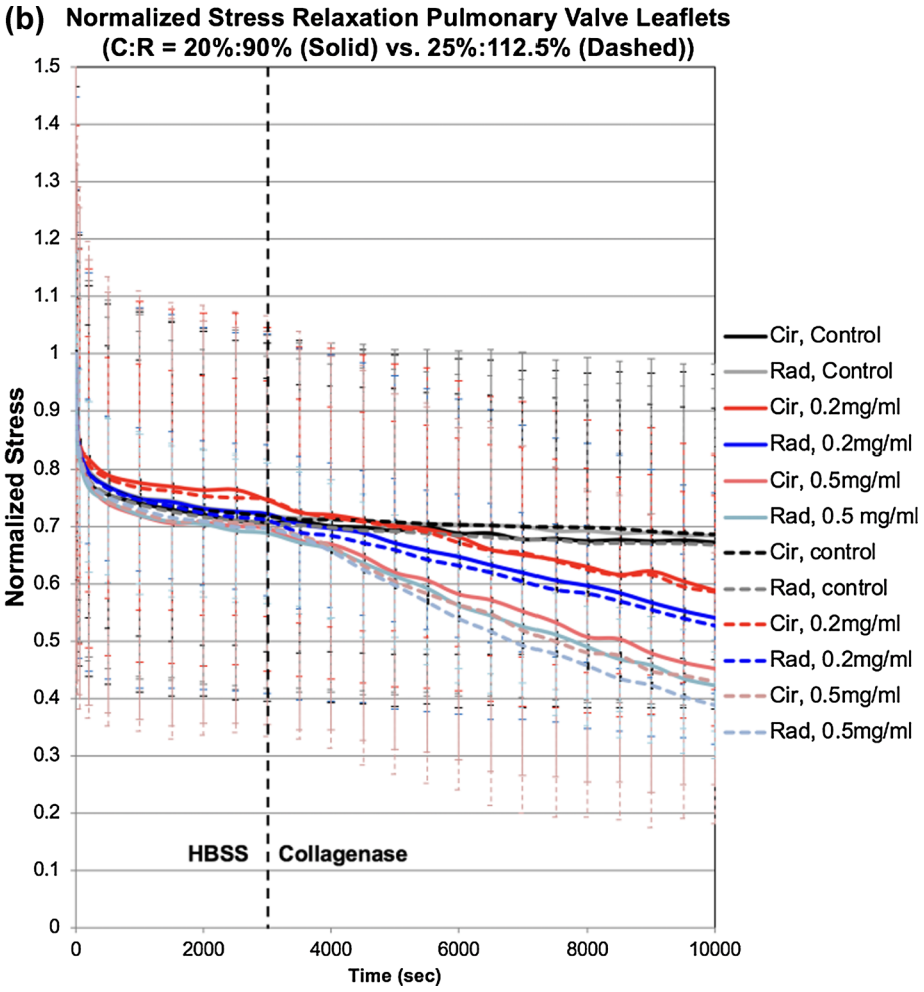

Fig. 6 (*Continued*)

collagen structure. In addition, creep testing and dynamic mechanical loading should be included to better understand the protection mechanisms under proteolysis at physiological strain states.

4 Conclusion

While MMPs-mediated collagen remodeling is recognized as a key aspect of valvular physiology and pathology and plays a significant role in bioprosthetic heart valve degeneration, it remains unclear how MMPs are capable of differentiating between damaged and undamaged collagen fibers, selectively degrading damaged collagen fibers and leaving undamaged, structurally load-bearing fibers unscathed. In this study, biaxial stress-relaxation behaviors in porcine heart valve leaflet tissues were investigated at physiological strain states. Based on previous studies by Adhikari et al. (2011), Huang and Huang (2015), Balachandran et al.

(2006), Anssari-Benam et al. (2011), it was suggested that a protective mechanism exists to prevent collagen degradation and that this mechanism was based on the applied strain. To this end, physiologically relevant strain states were imposed on AV and PV leaflets in combination with varying concentrations of collagenase to determine if there was a significant change in stress relaxation as compared with previous studies done at equibiaxial, non-physiological strain states. It was found that at physiological strain states, there was a significant decrease in the magnitude of stress relaxation as compared with equibiaxial testing for both AV and PV leaflets, even as the collagenase concentration increased. Histological images of the AV and PV using Masson's Trichrome suggested little change in the collagen fiber orientation. The images suggest that even when stretched to high physiological strain levels and exposed to increased concentrations of collagenase, the crimping of the collagen fibers still existed. There was no indication of collagen degradation, again suggesting a protective mechanism is present that prevents collagen catabolism when exposed to physiological strain states. From this study, based on biaxial stress-relaxation studies and histology, it can be concluded that there exists a protective mechanism to effectively block proteolytic sites on collagen when exposed to physiologically relevant strain states, conferring resistance to degradation.

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