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AN INVESTIGATION OF TRICUSPID VALVE LEAFLET DECELLULARIZATION AND

EFFECTS ON LEAFLET BIOMECHANICS AND COLLAGEN ARCHITECTURE

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AN INVESTIGATION OF TRICUSPID VALVE LEAFLET DECELLULARIZATION AND

EFFECTS ON LEAFLET BIOMECHANICS AND COLLAGEN ARCHITECTURE

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BY THE COMMITTEE CONSISTING OF

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Abstract

The Tricuspid Heart Valve is composed of three leaflets: the anterior leaflet, posterior leaflet, and septal leaflet. The function of this valve is to open during diastole to allow blood to flow from the atrium to the ventricle, then close during systole to prevent backwards flow of blood, called regurgitation. Valve regurgitation can decrease the effectiveness of the heartbeat and can lead to death over time. Two common heart valve replacements are mechanical heart valves and xenografts. These options have both shown clinical success, however no replacement currently meets the criteria for hemocompatibility, immunological tolerance, and the potential to grow and remodel itself. The decellularized tissue-engineered heart valve (TEHV) may be the key to achieving all of these goals. Decellularization has the potential to remove any immunogenic markers from the tissue while maintaining the complex microstructure that is vital for proper cell differentiation and remodeling. In this study, an H&E staining procedure was optimized for further use in the lab. Nine decellularization procedures with different exposure times to detergent and enzyme solutions were compared to find the optimal procedure. We found that 24-hour exposure to detergent and 12-hour exposure to enzymes was the optimal decellularization procedure for all three leaflets. This optimized decellularization procedure was then used in a biaxial mechanical and collagen microstructural analysis study to determine if the biaxial mechanical characteristics and collagen fiber architecture change as a result of the decellularization treatment. After statistical analysis of several parameters, we found that there were no statistically significant changes from the pre-treatment values to the post-treatment values due to decellularization reagent exposure. These results provide strong evidence that the chosen decellularization procedure is effective at decellularizing the tissue while maintaining the microstructure architecture and mechanical properties of the native leaflet.

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Chapter 1 - Introduction

The right atrioventricular heart valve, also known as the tricuspid valve, is located at the top of the right ventricle and serves as a gateway between the right atrium and right ventricle. It is composed of three collagenous leaflets which open toward the ventricle as blood rushes from the atrium to the ventricle during diastole. During systole, the right ventricle contracts to move blood through the pulmonary valve and into the pulmonary artery. The tricuspid valve closes during this contraction, preventing backflow of blood into the atrium. This backflow of blood is a condition known as heart valve regurgitation, which inhibits the effectiveness of the heartbeat, causing the heart to work harder. If left untreated, tricuspid valve regurgitation can lead to heart failure. The current options for heart valve replacement have many complications. Mechanical valves have poor hemocompatibility which puts the recipient at increased risk of blood clots, and biological replacements such as xenografts and homografts are likely to calcify or degenerate within ten years of implantation. Current valve replacement options do not grow with the body, so pediatric patients require a series of valve donations as their hearts grow.

1.1 Motivation

The tissue-engineered heart valve (TEHV) is a replacement option that has the potential for hemocompatibility, immunological compatibility, and the potential to grow and remodel itself. A valve that achieves these criteria could prevent the need for anticoagulants, immunosuppressants, or reoperation. There are two main types of TEHV scaffolds: decellularized tissue and synthetic polymer scaffolding. Decellularized xenograft heart valves maintain the complex microstructure of the native leaflets, which has an important influence on cell differentiation. However, removal of cellular and genetic material by chemical or

mechanical means has the potential to damage the extracellular matrix (ECM). Damage to the ECM has been characterized using histology and microscopy methods; however, there has not been analysis of how the microstructure behaves under pathologic loads post-decellularization. There is a need to characterize the collagen microstructure and quantify collagen alignment before and after decellularization treatment, as well as analyze the biaxial mechanical characteristics of the leaflets pre- and post-treatment.

1.2 Objective and Scope

The objective of this thesis is to analyze the effect of the chosen decellularization treatment on the biaxial mechanical characteristics of the tricuspid valve posterior leaflet. The studies performed to achieve this data are as follows:

1. Histology procedure optimization

    Several hematoxylin and eosin procedures were compared to achieve optimal images of the decellularized tissues.

2. Decellularization optimization of the three tricuspid valve leaflets

    In this study, a tricuspid valve leaflet was sectioned into 9 strips and exposed to a first solution of Triton X-100 and sodium deoxycholate, then a second solution of DNase and RNase. Strips were exposed to the solutions for 0, 12, and 24 hours. This experiment was repeated for the anterior, posterior, and septal leaflets.

3. Biaxial Mechanical characterization and collagen microstructural analysis pre- and post-treatment

    In this study, a tricuspid valve posterior leaflet was biaxially tested under pathological loading conditions. The tissue was placed under various forces in the circumferential and radial directions to simulate diseased conditions in vivo. Collagen alignment was

measured under these loading conditions using polarized spatial frequency domain imaging (pSFDI).

Chapter 2 of this thesis provides relevant background information such as the anatomy and function of the tricuspid heart valve, tricuspid valve pathology and current treatment options, and an overview of progress in the field of Tissue-Engineered heart valves. Chapter 3 details the H&E histology procedure comparison to determine the optimal deparaffinization and staining procedures. Chapter 4 presents the methods and results of the decellularization treatment on the three tricuspid leaflets, and Chapter 5 covers the methods and results of the biaxial characterization and pSFDI tests. Finally, Chapter 6 includes a discussion of the key findings from this thesis and future areas of investigation.

Chapter 2 - Background Information

2.1 Anatomy and Function of the Tricuspid Heart Valve

The tricuspid heart valve is located on the right side of the heart, and it is composed of three leaflets: the anterior leaflet, posterior leaflet, and septal leaflet. These leaflets are supported by the annulus, which connects them to the papillary muscles of the heart.
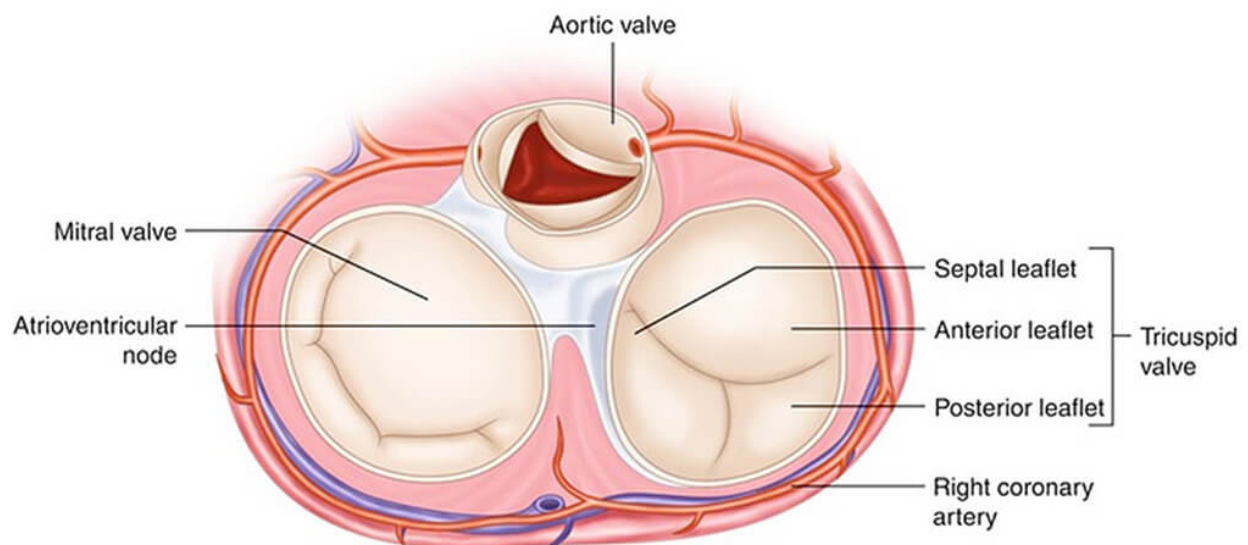
Figure 2-1. The Tricuspid Heart Valve. Image adapted from StatCardiologist.com.

The annulus is an elliptical ring of fibrous tissue, and it can change shape as the myocardium contracts during systole. The tricuspid valve's function is to move blood from the right atrium to the right ventricle, and to prevent regurgitation, or backflow, of blood into the atrium. The tricuspid valve leaflets are attached to the ventricular myocardium through collagenous fibers called chordae tendineae, which prevent the leaflets from opening toward the atrium during systole.
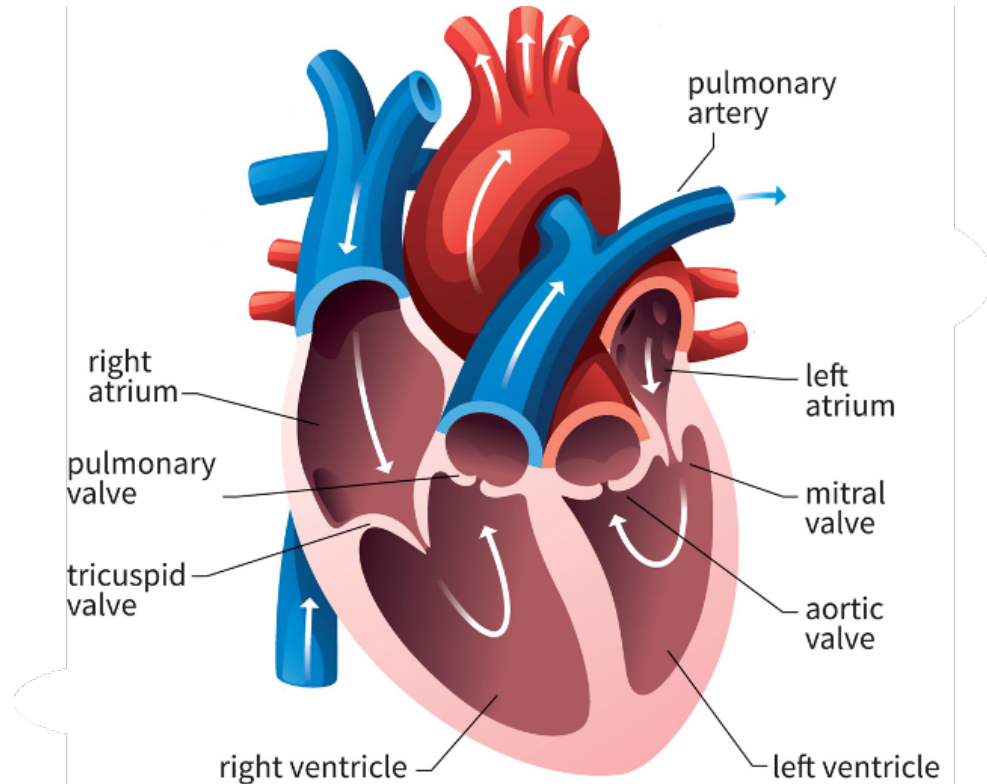
Figure 2-2. Heart Anatomy, including the valves and direction of blood flow. Image modified from cardiologyassociatesofmichigan.com.[1]

The tricuspid valve leaflet microstructure is primarily composed of collagen, elastin, and glycosaminoglycans, and all constituents are distributed heterogeneously. Tricuspid valve leaflets are separated into four distinct layers: the atrialis, spongiosa, fibrosa, and ventricularis. The atrialis is a monolayer of valvular endothelial cells (VECs) and the matrix is primarily composed of elastin.[2] The spongiosa is a layer of primarily glycosaminoglycans and proteoglycans, and is thought to act as lubricant between the atrialis and fibrosa layers.[3] The fibrosa is primarily composed of collagen fibers, and the ventricularis layer is composed of collagen and elastin.

2.2 Tricuspid Valve Pathology and Treatment Options

Tricuspid pathology has two classifications: primary pathology, due to an intrinsic valvular condition, and secondary pathology, which occurs as a result of disease elsewhere in the heart[4]. Primary tricuspid valve pathology can be congenital, as in Ebstein's anomaly, a disorder characterized by a fenestrated anterior leaflet and hypoplastic septal and posterior leaflets.[5] Primary pathology can also be acquired through endocarditis, radiation, damage due to cardiac device leads, and trauma, amongst others.[6] Tricuspid valve secondary pathology can result from disease in the left heart valves, myocardium, or pulmonary artery.[6]

One of the most common tricuspid valve pathologies is regurgitation, or the backward leakage of blood into the right atrium. Tricuspid regurgitation has been classified into two categories: functional and non-functional. Functional regurgitation is a direct result of pathology elsewhere in the heart, such as in left heart valve disease, myocardial disease, or pulmonary hypertension. Non-functional tricuspid regurgitation, or primary regurgitation, is due to damage of the tricuspid leaflets, annulus, chordae, or papillary muscles, and is seen less frequently.[7]

Another notable tricuspid pathology is stenosis, or narrowing of the valvular orifice. Rheumatic heart disease is a major cause of tricuspid stenosis, characterized by excessive fibrous thickening and fused valvular commissures.[8] This disease is an autoimmune reaction in response to infection by group A streptococcus, and leads to around 250,000 deaths per year globally.[9] Aside from rheumatic disease, stenosis can be caused by carcinoid tumor lesions, a pathology characterized by stiffened leaflets coated in plaque, obstructive vegetation due to infective endocarditis, congenitally underdeveloped tricuspid valves, or right ventricular tumors.[8]

Severe functional regurgitation is typically treated using surgical methods such as annuloplasty, a procedure designed to reduce annular diameter or reinforce annular geometry, as well as anterior leaflet enlargement, which involves replacement of the autologous anterior leaflet with a larger patch of pericardium. Leaflet stenosis due to primary valvular disease can be treated by replacement of affected area with a pericardial patch,[10] however, in many patients with valvular disease, valve damage is too extensive for surgical repair.[11] This creates the need for cost effective, readily available, and safe valve replacement options.

2.3 Tissue-Engineered Heart Valves

The ideal Tissue-Engineered heart valve should provide a scaffold for host cells to proliferate and remodel, integrating the graft into the host anatomy. There are currently two main types of scaffolds under investigation: decellularized tissue and synthetic polymer scaffolds.[12] Synthetic scaffolds do not require human or animal tissue donation and are therefore more readily available, however, a high degree of microstructural anisotropy is necessary to mimic native leaflet biomechanics.[13] Tissue scaffold decellularization preserves the complex network of collagen, elastin, and glycosaminoglycans, which has been shown to positively impact the differentiation of valvular interstitial cells (VICs),[14] a major component of native valve anatomy.

The primary drawback of xenograft scaffolds for Tissue Engineering applications is the potential for host immune response. Galactose-a1,3-Galactose, also known as a-Gal or Gal epitope, is a cell membrane antigen that has been identified as a major cause of inflammation and ultimate transplant rejection. It is estimated that up to 1% of human Immunoglobulin G (IgG) antibodies are anti-Gal as a result of constant exposure to a-Gal+ bacteria in the digestive tract.[15] Host anti-Gal antibodies binding to a-Gal epitopes upon transplantation activates the complement cascade which can destroy the xenograft in minutes, a condition known as

hyperacute rejection.[16] The depletion or neutralization of anti-Gal antibodies from the host blood stream in combination with immunosuppressive therapy can delay this response, however once the antibodies are replenished, the graft will be rejected immediately.[17] Even if hyperacute rejection can be avoided, acute humoral xenograft rejection (AHXR) can occur days later from a combination of antibody deposition and innate immune cell infiltration.[18] In addition to cell-surface proteins, foreign DNA and RNA have the potential to induce an immune response during xenotransplantation, however it has been shown clinically that small DNA fragments (<300 bp) are not enough to induce an immune response.[19] Removal of cell surface antigens and genetic material through decellularization techniques may be the key to xenotransplantation without the need for host immune suppression.

Xenografts implanted into the human body tend to exhibit one of two possible long-term remodeling responses. The first is characterized by chronic inflammation and fibrous encapsulation, and the second by organized and appropriate tissue remodeling.[20] Chemical crosslinking of collagen fibers within ECM scaffolds has been used as a method to increase durability of implanted tissue scaffolding over time, however this change in tissue topology has been shown to induce a pro-inflammatory phenotype (M1) in local macrophages, leading to chronic inflammation.[21, 22] By contrast, immunomodulatory macrophages (M2) induce proteolytic ECM degradation, which has been shown to release chemotactic signals which recruit multipotential progenitor cells to the scaffold site and trigger differentiation.[23, 24] Because the purpose of the TEHV is to be broken down and remodeled, chemical crosslinking was not employed in this study.

There are many methods of tissue decellularization, including the use of ionic, non-ionic, and zwitter-ionic detergent to lyse cell membranes, enzymes such as nucleases which cleave

genetic material, and mechanical methods such as osmotic shock, pressure gradients, radiation, and many others. In 2017, VeDepo *et al.* reported over the various methods of heart valve decellularization and included the decellularization effectiveness as well as the general effect of the procedure on the ECM. The most promising method was shown to be a combination of Triton X-100 and sodium deoxycholate, which showed complete lack of nuclei, 98% DNA removal, and histological preservation of the ECM.[12] This method, in combination with DNase and RNase treatment to reduce leftover genetic material, may be the key to producing a heart valve ECM scaffold that is hemocompatible, immunologically compatible, and readily remodeled by native host cells.

Chapter 3 - Histology Optimization

A robust procedure to differentiate between nuclei and other tissue components is necessary to establish the efficacy of the chosen decellularization method. Most tissues appear colorless and possess similar optical densities when studied under a light microscope,[25] so stain is introduced to differentiate between components. Anionic molecules such as nucleic acids are referred to as basophilic, meaning they bind with basic stains. Likewise, cationic molecules such as collagen and cytoplasmic components are acidophilic. Hematoxylin and Eosin staining, or H&E, is the most commonly used staining procedure due to its relatively simple procedure and inexpensive cost.[25] Hematoxylin targets acidic molecules such as nucleic acid, which renders nuclei dark purple when viewed under a microscope. A counterstain of Eosin Y binds to cytoplasm, collagen, and smooth muscle, dyeing the positively charged materials pink.[26]

There are two distinct H&E procedures referred to as progressive and regressive staining. With a progressive stain procedure, tissues are exposed to hematoxylin just long enough to stain anionic nuclei, then washed in water to remove unbound hematoxylin.[26] Regressive staining involves over-staining the tissue with hematoxylin, then removing excess hematoxylin with a differentiator such as acetic acid. This method is ideal for charged slides, which tend to induce background staining of non-nuclear material.[27]

Before tissues can be stained, embedded wax must be removed in a process called deparaffinization. Multiple xylene clearant baths can be used to remove paraffin wax. Xylene is insoluble in water, so excess clearant must be removed from the slide via alcohol baths starting at a concentration of 100% and subsequently decreasing.[26] Then, tissues are hydrated in a water bath before staining with aqueous hematoxylin.

In this study, two deparaffinization protocols were compared with a progressive stain procedure and a regressive stain procedure to determine the optimal combination.

3.1 Methods

Each leaflet was sectioned into three circumferential strips, and strips were fixed in 10% neutral buffered formalin for 24 hours. Paraffin infiltration was performed according to the procedure in Table 3-1.

Table 3-1. Tissue exposure time for each reagent of the paraffin infiltration procedure.

Reagent	Time (minutes)
70% Alcohol	30
95% Alcohol	30
100% Alcohol	30
100% Alcohol	30
100% Alcohol	30
100% Alcohol	60
Xylene	30
Xylene	15
Xylene	15
Wax	30
Wax	60

Tissues were then embedded into paraffin blocks and sectioned into 5-micron ribbons. Paraffin ribbons were placed in a water bath at 37 °C to smooth wrinkles in the section, then sections were floated onto charged glass slides. Two sections were adhered to each slide, and two slides were made for each tissue strip.

Four test groups were implemented to determine the optimal deparaffinization and staining procedure. The test groups were labeled A, B, C, and D, and the procedures are listed in Table 3-2.

Table 3-2. Deparaffinization and staining procedures. Procedures A and B are progressive procedures, and Procedures C and D are regressive procedures.

A		B		C		D	
3 mins	xylene	2 mins	xylene	2 mins	xylene	3 mins	xylene
3 mins	xylene	2 mins	xylene	2 mins	xylene	3 mins	xylene
3 mins	100% ethanol	2 mins	100% ethanol	2 mins	100% ethanol	3 mins	100% ethanol
3 mins	90% ethanol	2 mins	100% ethanol	2 mins	100% ethanol	3 mins	90% ethanol
3 mins	70% ethanol	2 mins	95% ethanol	2 mins	95% ethanol	3 mins	70% ethanol
rinse	water	2 mins	water	2 mins	water	rinse	water
5 mins	hematoxylin	5 mins	hematoxylin	3 min	hematoxylin	3 min	hematoxylin
rinse	DI water	rinse	DI water	1 min	water	1 min	water
rinse	DI water	rinse	DI water	1 min	acetic acid	1 min	acetic acid
10-15 s	Blueing	10-15 s	Blueing	1 min	water	1 min	water
rinse	DI water	rinse	DI water	1 min	Blueing	1 min	Blueing
rinse	DI water	rinse	DI water	1 min	water	1 min	water
rinse	100% ethanol	rinse	100% ethanol	1 min	95% ethanol	1 min	95% ethanol
2-3 mins	Eosin Y	2-3 mins	Eosin Y	45 sec	eosin Y	45 sec	eosin Y
rinse	100% ethanol	rinse	100% ethanol	1 min	95% ethanol	1 min	95% ethanol
rinse	100% ethanol	rinse	100% ethanol	1 min	100% ethanol	1 min	100% ethanol
rinse	100% ethanol	rinse	100% ethanol	1 min	100% ethanol	1 min	100% ethanol
rinse	100% ethanol	rinse	100% ethanol	2 mins	xylene	2 mins	xylene
				2 mins	xylene	2 mins	xylene

Procedures C and D are regressive stain procedures adapted from Leica Biosystems.[27] After completion of staining, slides were allowed to dry for 5 minutes, then mounted with DPX

mountant and coverslips were applied. Slides were imaged using a microscope under 10x

magnification.

3.2 Results

10x magnified images of progressively stained A and B for all three tricuspid leaflets

showed dark background staining and poor contrast between nuclei and collagen, whereas

regressively stained C and D showed light pink collagen staining with good contrast. There were

no obvious differences between groups A and B or between groups C and D, suggesting that

both the shorter and the longer deparaffinization procedures are effective.
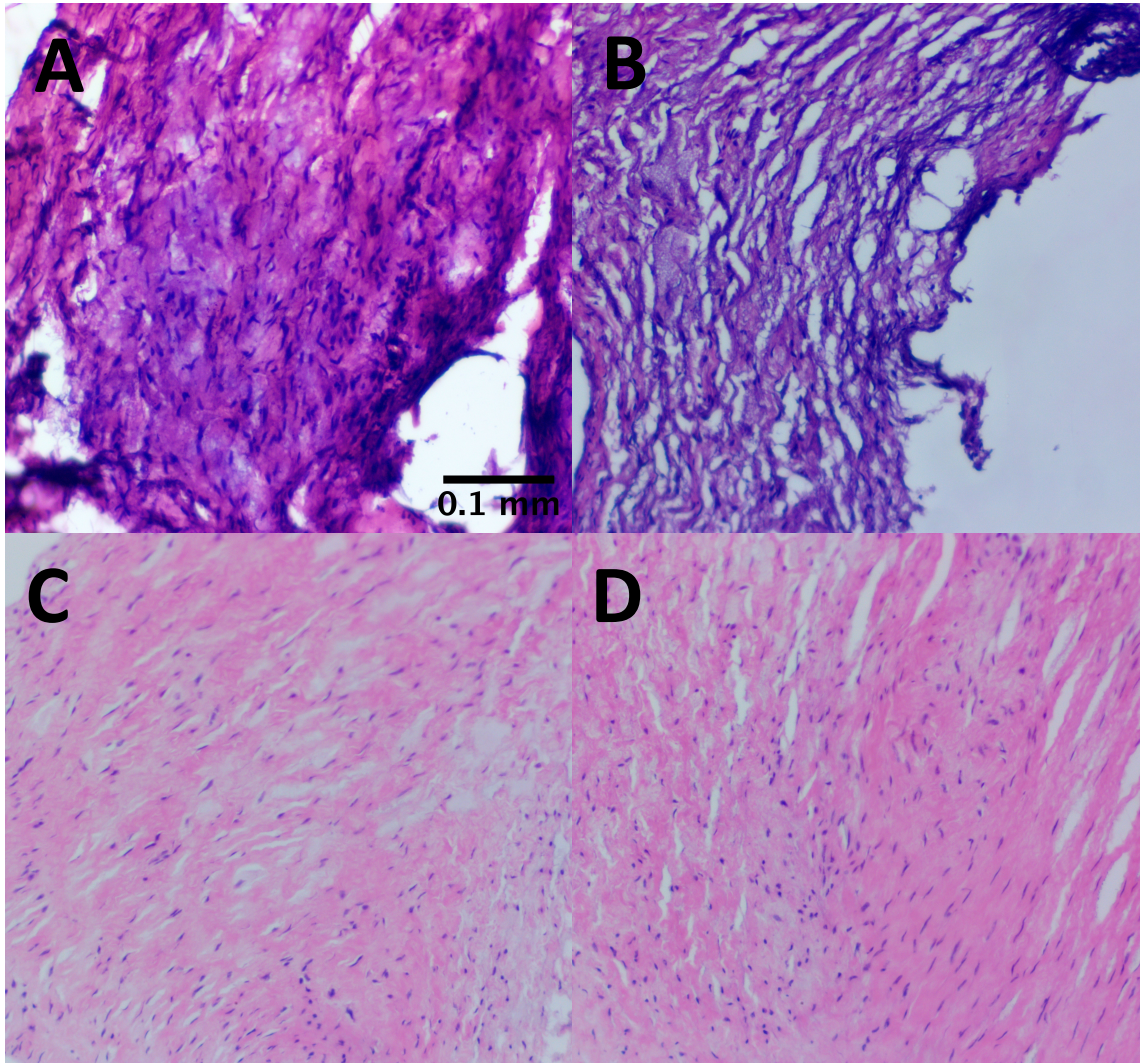
Figure 3-1. 10X magnification of TVPL stained with H&E. Samples A and B were stained with a progressive procedure. Samples C and D were stained with a regressive procedure. Collagen is stained pink, nuclei are stained purple.
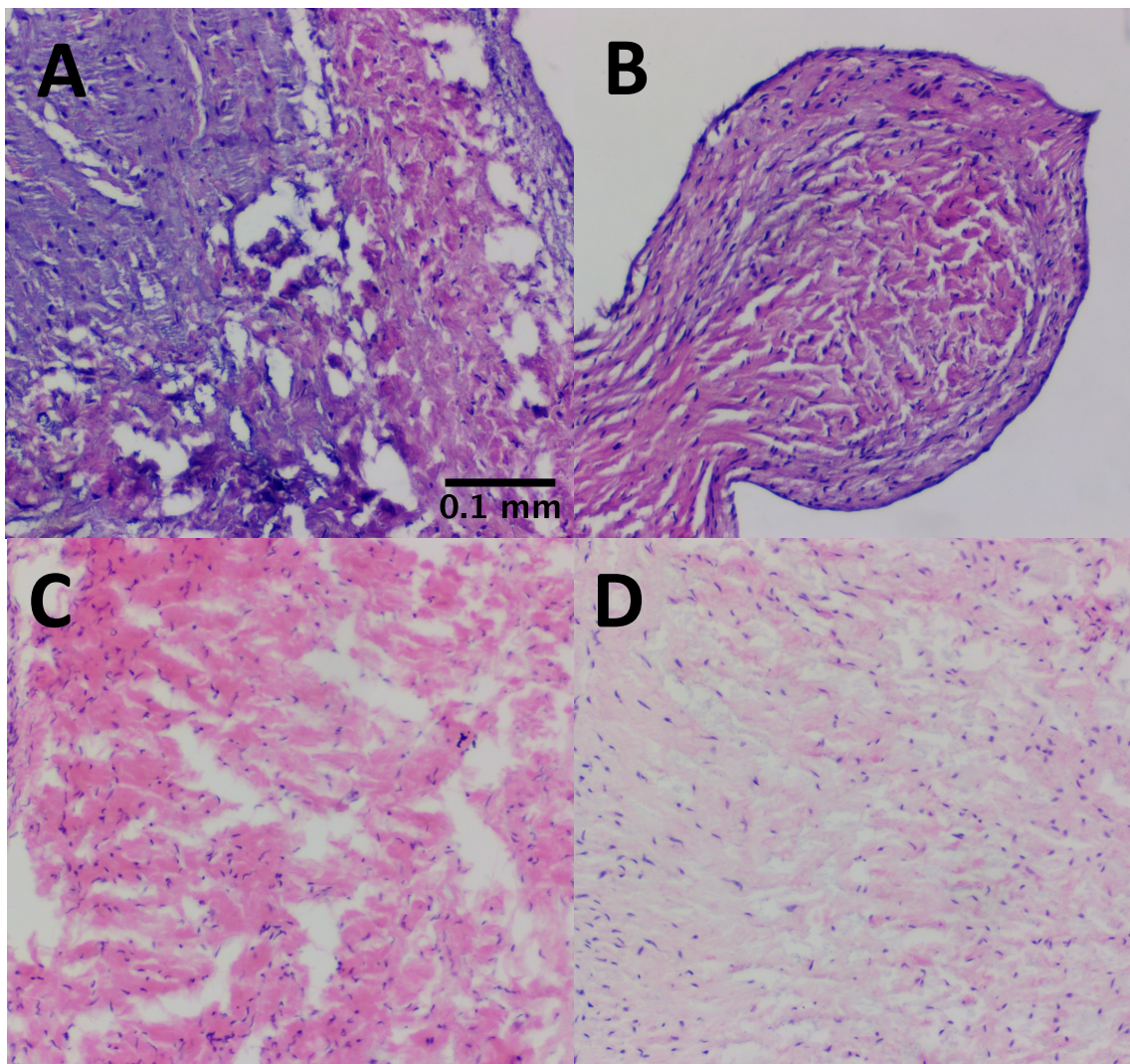
Figure 3-2. 10X magnification of TVAL stained with H&E. Samples A and B were stained with a progressive procedure. Samples C and D were stained with a regressive procedure. Collagen is stained pink, nuclei are stained purple.
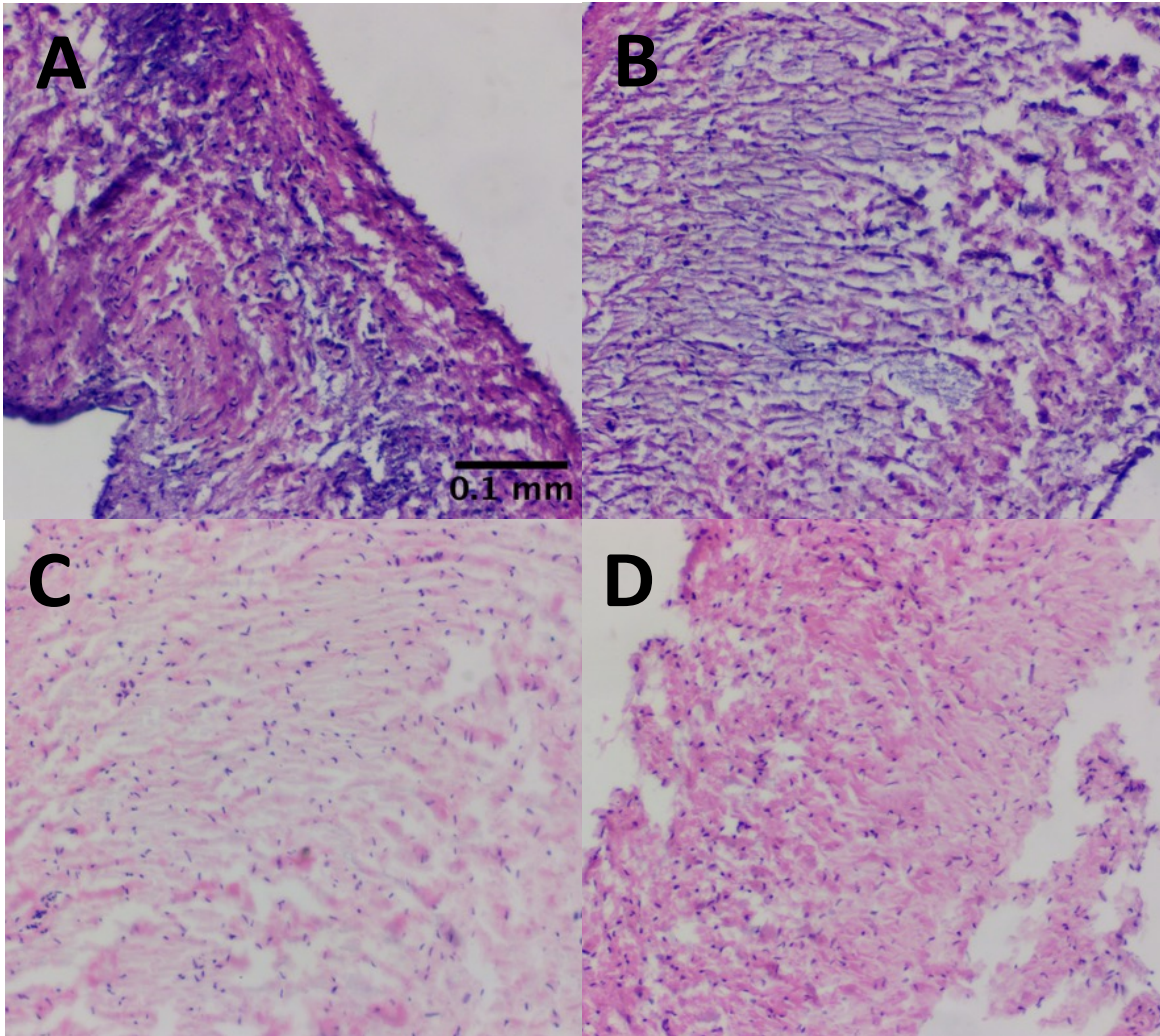
Figure 3-3. 10X magnification of TVSL stained with H&E. Samples A and B were stained with a progressive procedure. Samples C and D were stained with a regressive procedure. Collagen is stained pink, nuclei are stained purple.

3.3 Discussion

Variation between labs and between lab workers, as well as many other factors such as stain oxygenation, solution contamination or dilution, solution pH, and tissue fixation quality frequently cause inconsistent histology results (Feldman). Results from this procedure may vary between individuals and between tissue samples. However, the results of this study consistently showed that regressively stained Tricuspid Valve leaflets show no hematoxylin background staining, as well as good contrast between nuclei and collagen. Additionally, there were no noticeable differences between longer and shorter deparaffinization times.

Treatment C (regressive stain, short deparaffinization time) was chosen as the optimal H&E staining procedure for this lab, and this procedure was used to determine the effectiveness of the decellularization study described in Chapter 4.

Chapter 4 - Decellularization

In order to test the biaxial mechanical characteristics and collagen alignment of decellularized Tricuspid Valve Posterior Leaflets, a decellularization procedure was optimized to find the minimum treatment time necessary to remove cellular and genetic material with minimal damage to the Extracellular Matrix (ECM). Previous studies have reported that detergents Triton X-100 and sodium deoxycholate effectively decellularize heart valve tissue with minimal ECM damage, however no study has compared different exposure times to find minimum exposure time necessary to decellularize the tissue.[28, 29, 30, 31, 32]

4.1 Methods

Three porcine hearts were acquired from a local slaughterhouse and were dissected to excise the tricuspid valve leaflets, for a total of three tricuspid valve anterior leaflets (TVALs), three tricuspid valve posterior leaflets (TVPLs), and three tricuspid valve septal leaflets (TVSLs). Tissues were stored at 4 °C until the time of the experiment, then were thawed with 50 mM MgCl2 PBS. Leaflets were sectioned into 9 circumferentially oriented strips, with the first strip closest to the annulus (Figure 4-1).

Figure 4-1. TVSL sectioned into 9 circumferentially oriented strips.

A detergent solution of 0.05% Triton X and 1% w/v sodium deoxycholate was made by adding 0.5 mL Triton X and 0.01 g sodium deoxycholate to 999.5 mL deionized water. An enzymatic solution of 100 ug/mL RNase and 0.2 mg/mL DNase was synthesized by adding 5 mg RNase and 10 mg DNase to 50 mL PBS with 50 mMol MgCl2.

Nine testing conditions were identified to determine the optimal combination of detergent and enzyme exposure. Tissues were exposed to combinations of 0, 12, or 24 hours of detergent and 0, 12, or 24 hours of enzymes. The exposure times for each tissue is presented in Table 4-1.

Table 4-1. Detergent exposure time (hours) and Enzyme exposure time (hours) for each tissue sample.

Tissue	Detergent Exposure Time (h)	Enzyme Exposure Time (h)
1	0	0
2	0	12
3	0	24
4	12	0
5	12	12
6	12	24
7	24	0
8	24	12
9	24	24

Immediately after the leaflets were sectioned, each tissue strip was placed in a microvial with detergent at room temperature for the designated time. After exposure to detergent, tissues were washed in DI water for 24 hours at room temperature to remove residual detergent. After

the DI water wash, each tissue was moved into a microvial with the enzyme solution for the

designated time and maintained at 37 °C. Enzyme exposure was followed by a 24-hour wash in

PBS at 37 °C. The steps of this procedure are shown in Figure 4-2. Immediately after the PBS

bath, tissues were fixed with 10% neutral buffered formalin for 24 hours, and then stored in a

70% ethanol solution. This procedure was repeated for the TVAL, TVSL, and TVPL with *n*=3

for each leaflet.

| Detergent Solution | → | DI water wash | → | Enzymatic Solution | → | PBS wash |
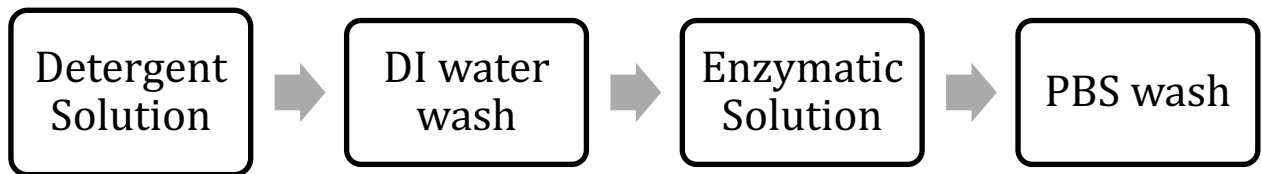
Figure 4-2. Steps of the decellularization procedure.

Tissues were stained with Hematoxylin and Eosin procedure detailed in section 3, and

images were taken under 10x magnification. In addition to H&E staining, Alcian Blue staining

was used to qualitatively examine the effects of the decellularization procedure on the GAG

content in the leaflet. Sirius Red and Trichrome staining were used to examine the collagen

content in response to the decellularization procedure, and pentachrome was used to view other

components such as elastin, which stains purple.
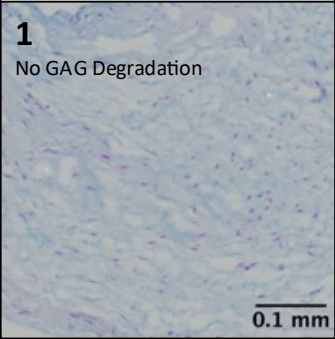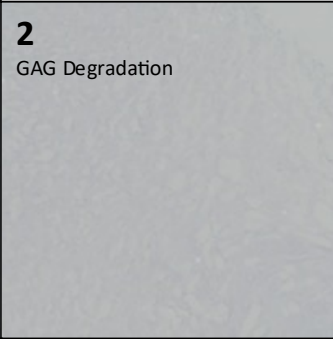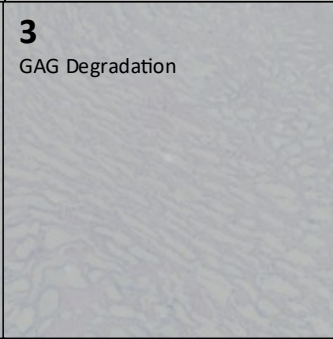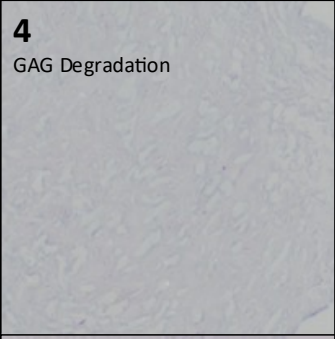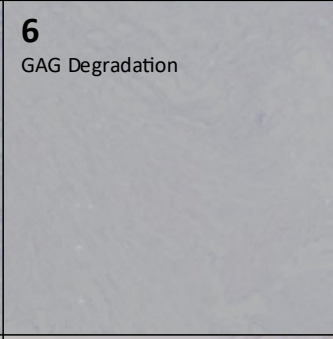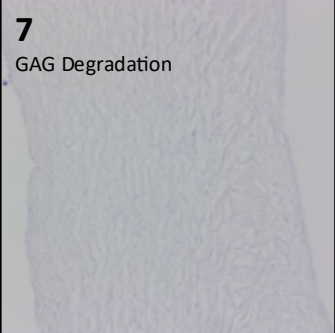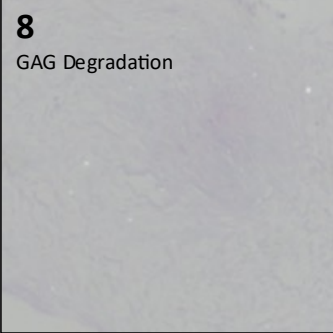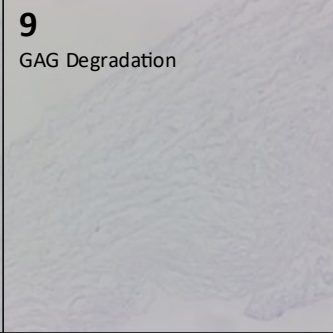
4.2 Results- TVSL

Images taken from H&E-stained tissues showed no decellularization in Tissue 1 (0 h detergents, 0 h enzymes). Tissues 2–7 showed partial decellularization, with a lower frequency of cells left in the ECM than in un-decellularized tissues. Tissues 8 and 9 showed complete decellularization with no visible cells in the extracellular matrix.

	Enzyme Exposure 0 h	Enzyme Exposure 12 h	Enzyme Exposure 24 h
Detergent Exposure 0 h	**1** No Decellularization — 0.1 mm	**2** Partial Decellularization	**3** Partial Decellularization
Detergent Exposure 12 h	**4** Partial Decellularization	**5** Partial Decellularization	**6** Partial Decellularization
Detergent Exposure 24 h	**7** Partial Decellularization	**8** Complete Decellularization	**9** Complete Decellularization

Figure 4-3. 10X magnification of H&E-stained tissues. Collagen is stained pink, and nuclei are stained purple.

Alcian Blue stained tissues showed no GAG degradation in the control tissue (tissue 1), but visible GAG degradation in every other group tested.

	Enzyme Exposure 0 h	Enzyme Exposure 12 h	Enzyme Exposure 24 h
Detergent Exposure 0 h	**1** No GAG Degradation	**2** GAG Degradation	**3** GAG Degradation
Detergent Exposure 12 h	**4** GAG Degradation	**5** GAG Degradation	**6** GAG Degradation
Detergent Exposure 24 h	**7** GAG Degradation	**8** GAG Degradation	**9** GAG Degradation

Figure 4-4. 10X magnification of Alcian Blue-stained tissues. GAGs are stained blue, and nuclei are stained purple.

Sirius Red stained tissues were imaged at magnification of 4X to visualize the collagen network of a greater area of the leaflet. No collagen damage was visible in the control group or any of the eight treatment groups.
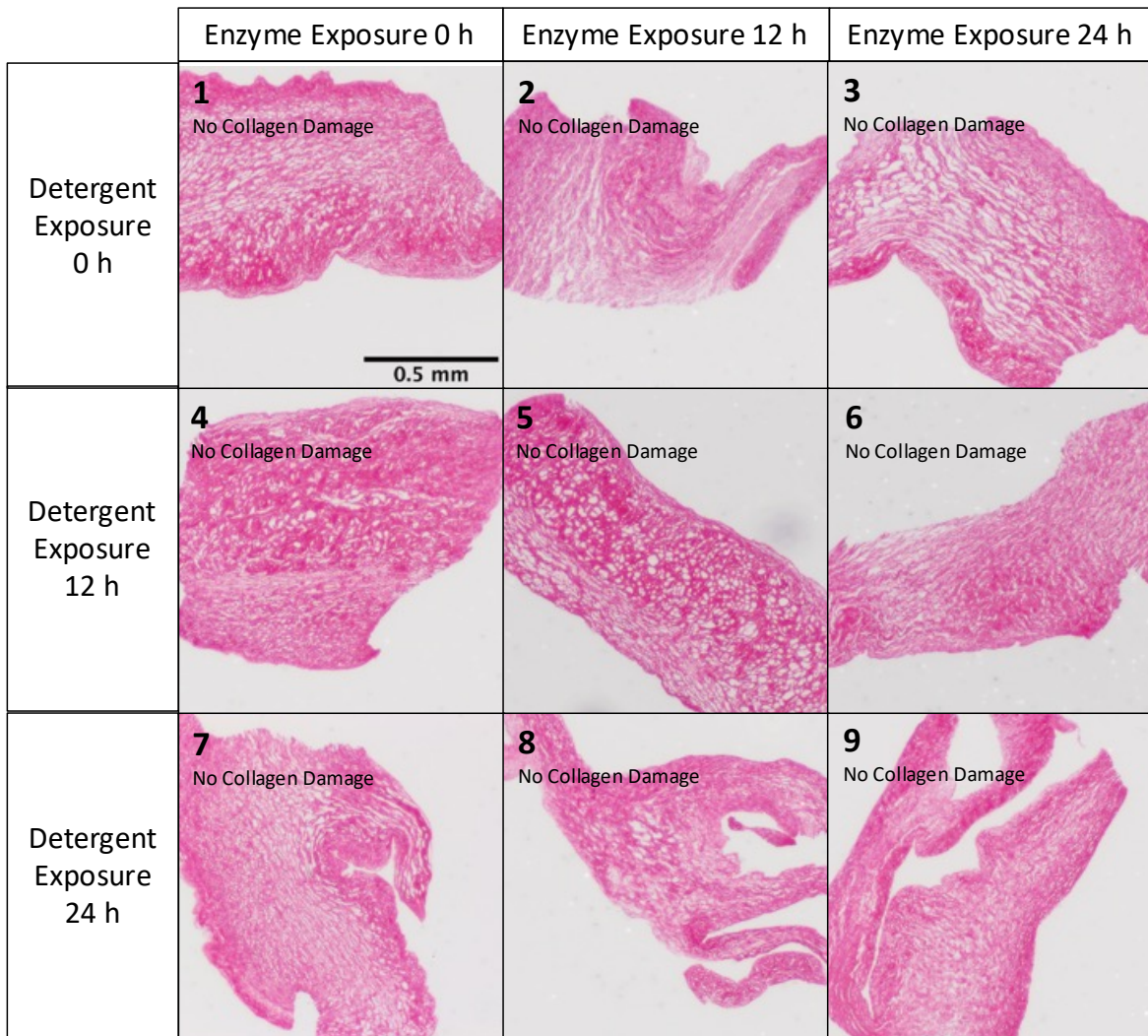
Figure 4-5. 4X magnification of Sirius Red-stained tissues. Collagen is stained dark pink.

Trichrome Stained tissues were imaged at 4X magnification, and no collagen damage was visible in the control group or treatment groups.

	Enzyme Exposure 0 h	Enzyme Exposure 12 h	Enzyme Exposure 24 h
Detergent Exposure 0 h	1 No Collagen Damage 0.5 mm	2 No Collagen Damage	3 No Collagen Damage
Detergent Exposure 12 h	4 No Collagen Damage	5 No Collagen Damage	6 No Collagen Damage
Detergent Exposure 24 h	7 No Collagen Damage	8 No Collagen Damage	9 No Collagen Damage
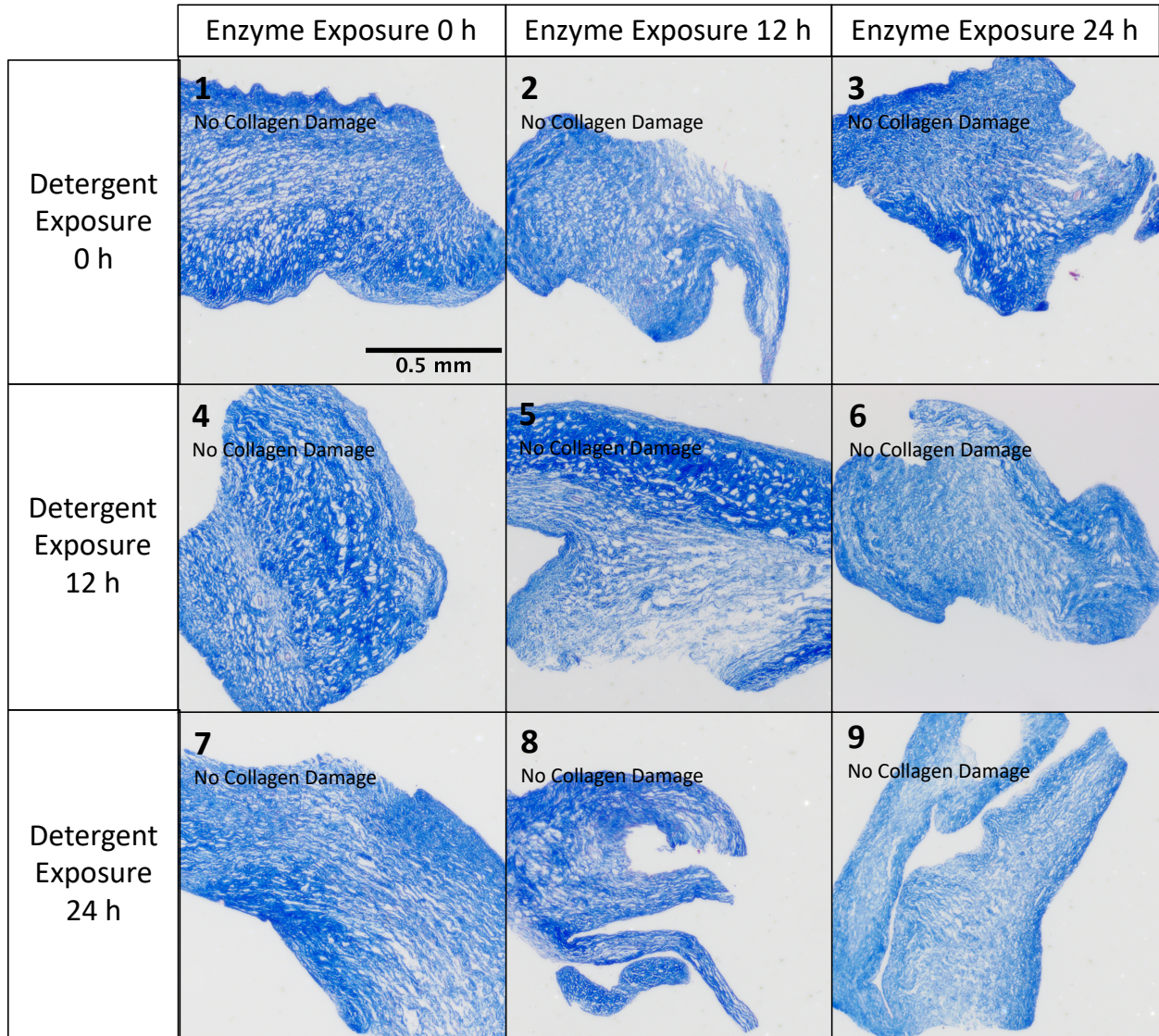
Figure 4-6. 4X magnification of Trichrome-stained tissues. Collagen is stained blue.

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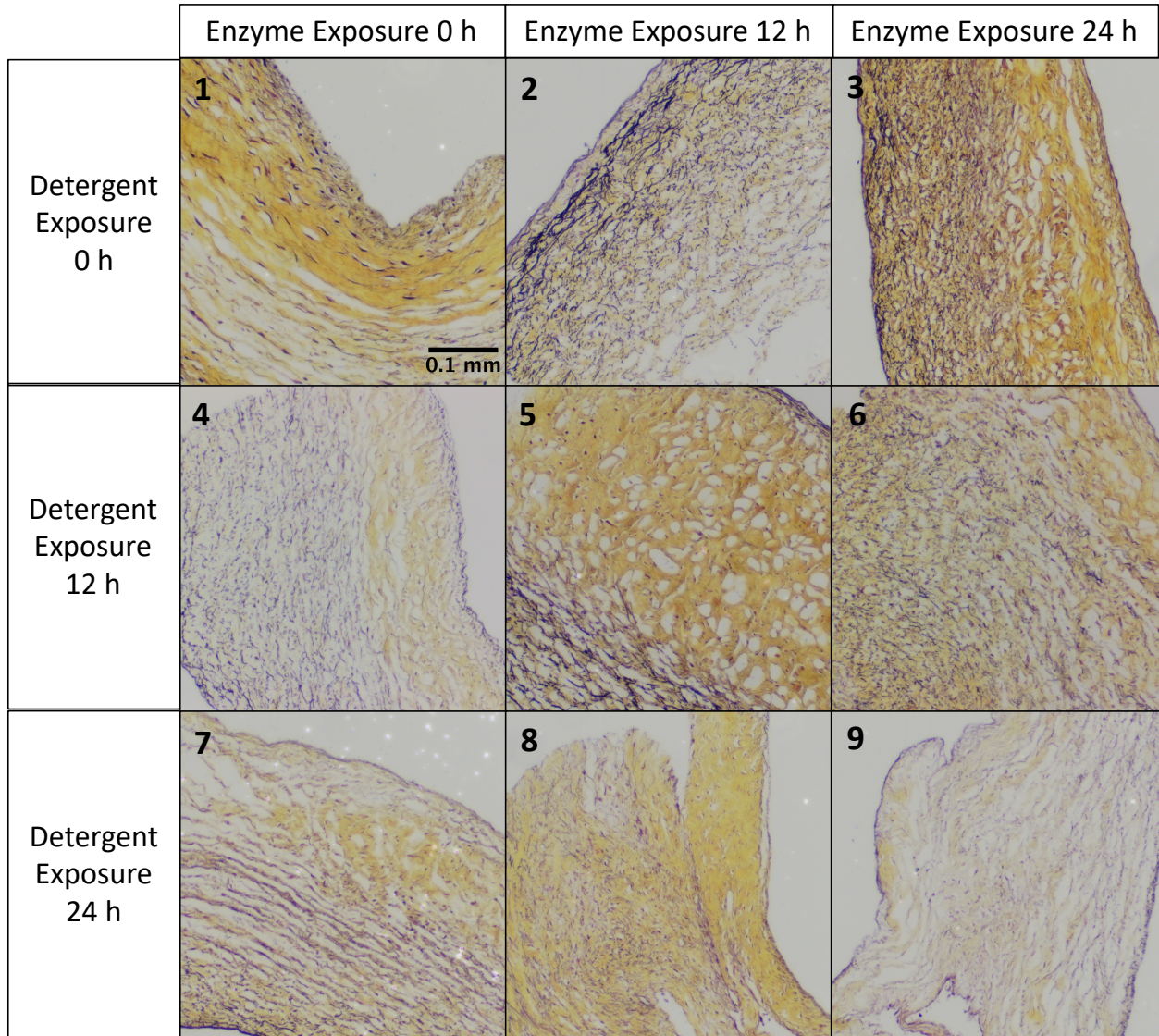
Figure 4-7. 10x magnification of Pentachrome-stained tissues. Nuclei and elastin are stained black. Collagen is stained yellow.
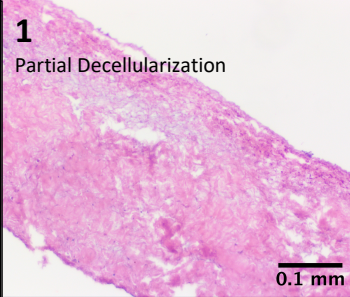
4.3 Results- TVAL

Decellularized TVAL leaflets underwent in-house H&E staining. TVAL tissues appear to decellularize more readily than TVSL tissues.

	Enzyme Exposure 0 h	Enzyme Exposure 12 h	Enzyme Exposure 24 h
Detergent Exposure 0 h	**1** No Decellularization	**2** Complete Decellularization	**3** Complete Decellularization
Detergent Exposure 12 h	**4** No Decellularization	**5** Complete Decellularization	**6** Complete Decellularization
Detergent Exposure 24 h	**7** Complete Decellularization	**8** Complete Decellularization	**9** Complete Decellularization

Figure 4-8. 10X magnification of H&E-stained tissues. Collagen is stained pink, and nuclei are stained purple.

4.4 Results- TVPL

|	Enzyme Exposure 0 h	Enzyme Exposure 12 h	Enzyme Exposure 24 h
Detergent Exposure 0 h	**1** Partial Decellularization	**2** Complete Decellularization	**3** Complete Decellularization
Detergent Exposure 12 h	**4** Partial Decellularization	**5** Complete Decellularization	**6** Complete Decellularization
Detergent Exposure 24 h	**7** Complete Decellularization	**8** Complete Decellularization	**9** Complete Decellularization

Figure 4-9. 10X magnification of H&E-stained tissues. Collagen is stained pink, and nuclei are stained purple.

4.5 Discussion

Histological results from the decellularization study consistently showed complete decellularization with no visible cell nuclei for both the TVAL and TVSL leaflets after decellularization protocol 8 (24-hour detergent exposure, 12-hour enzyme exposure) and protocol 9 (24 hour detergent exposure, 24 hour enzyme exposure). Protocol 8 was selected as the optimal decellularization treatment because a shorter treatment time is more ideal to prevent unnecessary ECM damage.

Trichrome and Sirius Red stained TVSLs showed no visible collagen damage. However, all TVSL decellularization groups showed visible GAG reduction compared to the control. Therefore, this treatment does not perfectly preserve the ECM of the leaflet.

Chapter 5 - Analysis of Mechanical Characteristics and Collagen Alignment of Decellularized Tissues

In 2020, Meador et al. characterized the behavior of all three tricuspid valve leaflets under biaxial mechanical tension. Fibrous soft tissues exhibit a J-shaped stretch response which can be characterized by an initial linear stretch at low membrane tension, a non-linear transition period in which collagen fibers uncrimp and engage, and an almost vertical linear segment with little tissue stretch. Meador's study showed consistent results for all three leaflets in the circumferential direction, but not in the radial direction, suggesting some mechanical anisotropy between the leaflets.[33]

Tricuspid leaflet collagen fiber network response to physiological and pathological loading scenarios has been investigated in-depth, but the effects of decellularization on these properties has never been reported. Changes in the collagen fiber architecture due to decellularization methods could provide evidence as to how the engineered leaflets will behave *in vivo*. Polarized spatial frequency domain imaging (pSFDI) provides insight into the alignment of collagen fibers in the leaflet through two values of interest: the θ_{Fiber}, which is the mean angle of a group of fibers, and the degree of optical anisotropy (DOA), or the dispersion of local collagen fibers (Figure 5-1).
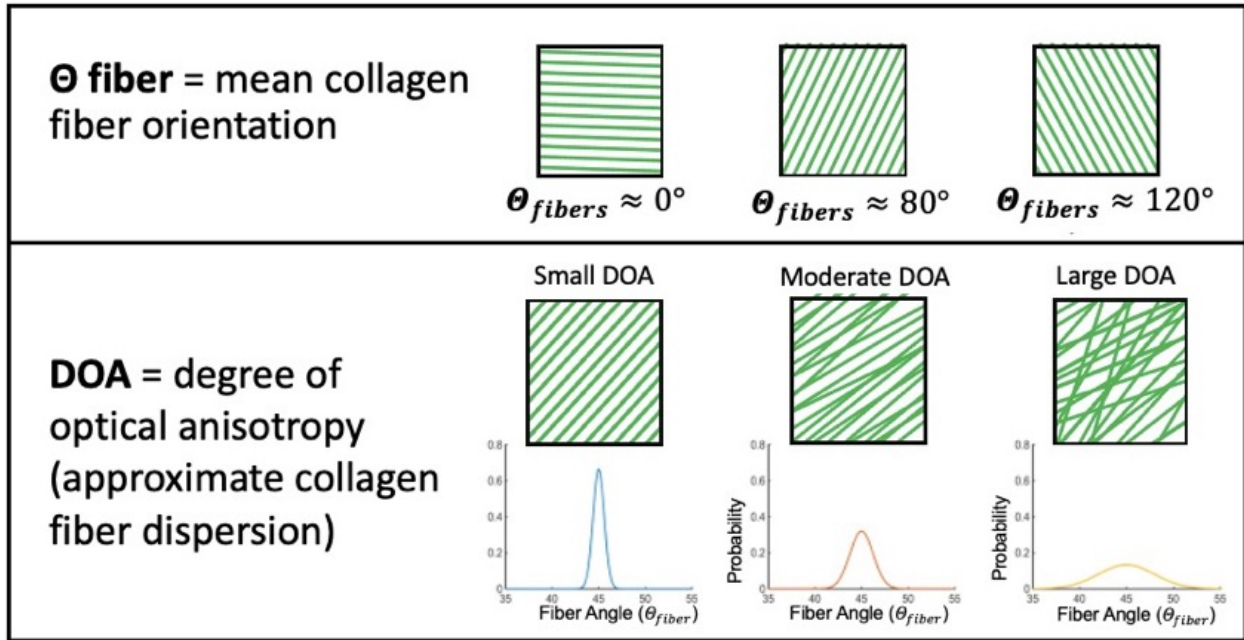
Figure 5-1. Representation of θ_{Fiber} and DOA parameters.

pSFDI analysis of collagen architecture employs birefringent collagen scattering, in which light is passed through a polarizer at an angle $\theta_{Polarizer}$, reflected from the tissue back through the same polarizer, and the intensity of the reflected light is measured[34] (Figure 5-2).
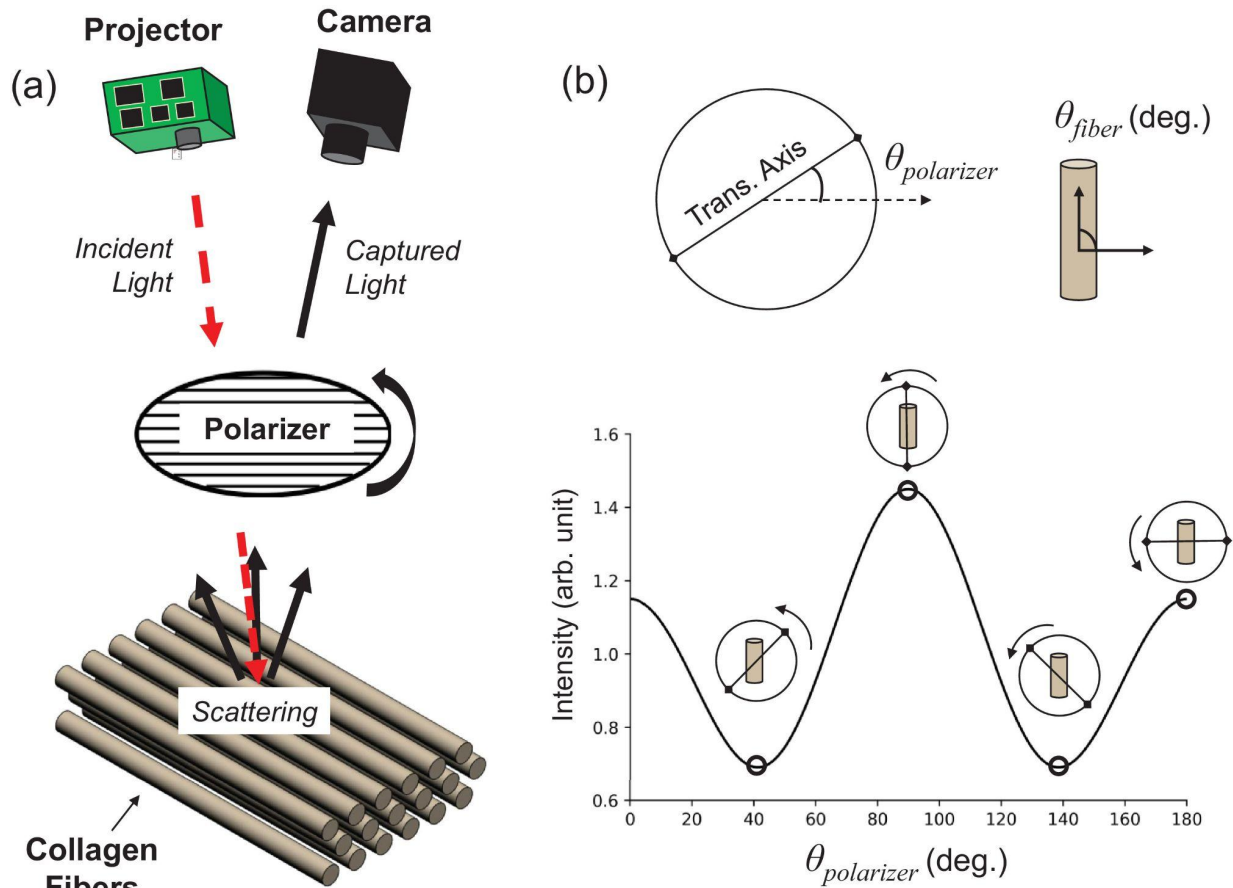
Figure 5-2. a) Schematic of system setup. b) Depiction of $\theta_{Polarizer}$ and θ_{Fiber}, as well as bimodal intensity peak. (Image from Jett et al. 2021[34])

The polarizer lens is rotated 180° and the intensity is measured every 5°, and results are represented in a graph showing measured light intensity vs. $\theta_{Polarizer}$. The peak measured intensity occurs when $\theta_{Fiber} = \theta_{Polarizer}$, meaning that the polarized light is parallel with the collagen fibers. The smaller peak occurs when $\theta_{Fiber} = \theta_{Polarizer} + 90°$, meaning polarized light is perpendicular to collagen fibers.

5.1 Methods

In this study, the biaxial mechanical characteristics and collagen alignment of TVPLs were analyzed before and after the decellularization procedure that was optimized in chapter 4. Leaflets were mounted to the biaxial tester with four rakes to form a testing square of 7.5 mm and submerged in PBS for the duration of the procedure. The circumferential direction of the leaflet was aligned with the X direction, while the radial leaflet aligned with the Y direction. Leaflets underwent 10 preconditioning stretch cycles to return them to their *in vivo* configuration, then were subjected to seven different loading conditions with different ratios of circumferential to radial forces (1:1, 1:0.75, 1:0.5, 1:0.25, 0.75:1, 0.5:1, 0.25:1). Tissue stretch in response to these stresses was collected. With the tissue still mounted to the biaxial tester, pSFDI was used to evaluate the collagen alignment under different circumferential to radial force ratios (unloaded, 1:1, 1:0.25, 0.25:1).

Immediately after biaxial and pSFDI characterization, experimental group tissues underwent the optimized decellularization procedure of four parts:

1. 24 hours in 0.05% Triton X and 1% w/v sodium deoxycholate

2. 24-hour DI water wash

3. 12-hour exposure to 100 ug/mL RNase and 0.2 mg/mL DNase

4. 24-hour PBS wash

Control group tissues underwent an extensive washing procedure to eliminate the possibility that observed results could be due to prolonged periods in solution:

1. 24 hours in DI water

2. 24 hours in DI water

3. 12 hours in PBS

4. 24 hours in PBS

Immediately following the final washing step for both experimental and control groups, tissues were remounted to the biaxial tester with a testing size of 6.5x6.5 mm to avoid the presence of tine holes in the testing region. The tissues underwent preconditioning, biaxial testing, and pSFDI characterization according to the same procedure described above (Figure 5-3).

| Biaxial and pSFDI Analysis | → | Decellularization Procedure | → | Biaxial and pSFDI Analysis |
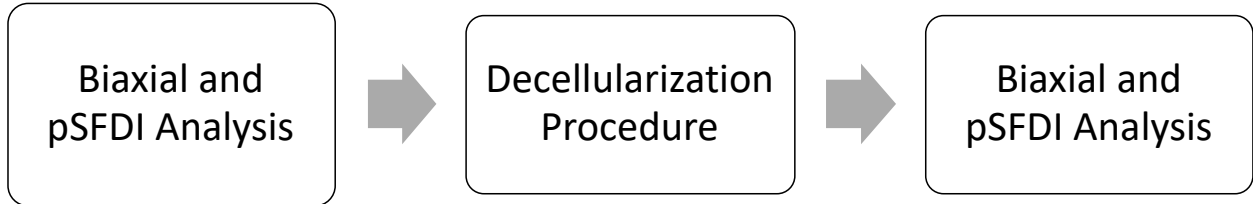
Figure 5-3. Summary of the biaxial/pSFDI, decellularization, biaxial/pSFDI pipeline.

A summary of the Biaxial/pSFDI analysis of decellularized tissues and control group tissues is shown in Table 5-1. This procedure was repeated with *n*=5 for each group.

Table 5-1. Summary of the decellularized group procedure vs. the control group procedure.

Decellularized Group		Control Group	
~3 h	Biaxial/pSFDI testing	~3 h	Biaxial/pSFDI testing
24 h	Solution 1	24 h	DI water wash
24 h	DI water wash	24 h	DI water wash
12 h	Solution 2	12 h	PBS
24 h	PBS wash	24 h	PBS wash
~3 h	Biaxial/pSFDI testing	~3 h	Biaxial/pSFDI testing

5.2 Results- Biaxial Testing

Several parameters were evaluated to discover whether the decellularization treatment has a statistically significant impact on the biaxial mechanics and collagen alignment of the TVPL. These parameters include high tension and low-tension moduli in both the circumferential and radial directions, peak stretch in both the circumferential and radial directions, average θ_{Fiber} across the tissue, and average DOA across the tissue. The high-tension modulus of the tissue is the approximated slope of the high tension, linear portion of the tension-stretch curve that is correlated with full collagen engagement and stretching. The low-tension modulus is the approximated slope of the low-tension linear region, correlated with collagen fiber uncrimping (Figure 5-4).
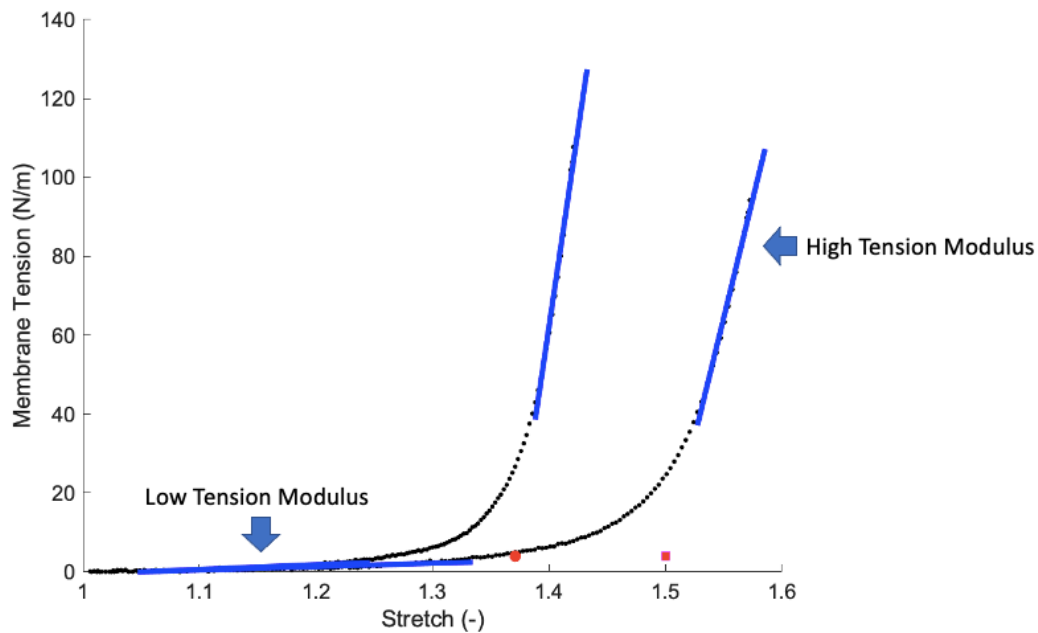
Figure 5-4. Membrane tension-stretch curve with high-tension moduli and low-tension moduli fitted to both the circumferential curve and the radial curve.

For each of the seven loading ratios (T_{circ}:T_{rad} = 1:1, 1:0.75, 1:0.5, 1:0.25, 0.75:1, 0.5:1, 0.25:1), the pre-treatment value and the post-treatment value for the low-tension modulus in the circumferential direction were compared to the using a paired t test. No statistically significant results were found from pre-treatment to post-treatment for any of the loading ratios, for either the control group or the decellularized group (Figure 5-5).
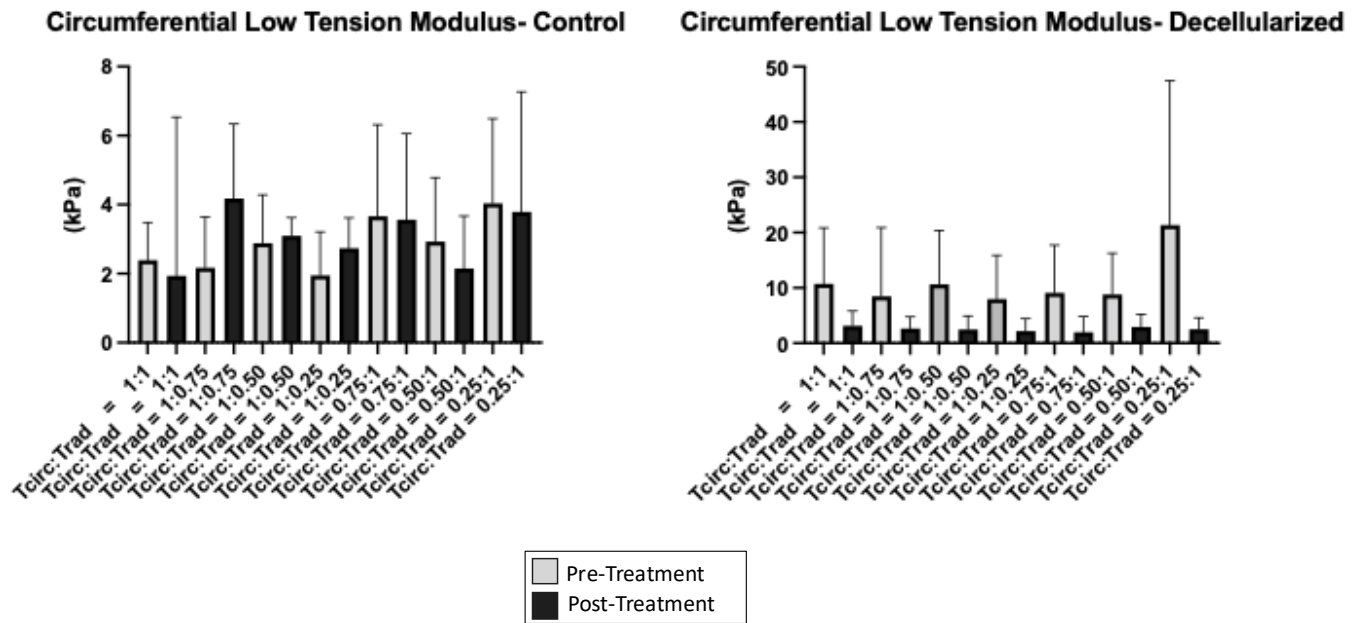
Figure 5-5. Circumferential low-tension modulus for control and decellularized treatment groups. Pre-treatment values are compared to post-treatment values with a paired t test. Statistical significance is indicated with $* = p < 0.05$, and $** = p < 0.005$.

Additionally, for the low-tension modulus in the circumferential direction, the post-treatment value for the control group was compared to the post-treatment value for the decellularized group for each loading protocol ($T_{circ}:T_{rad}$ = 1:1, 1:0.75, 1:0.5, 1:0.25, 0.75:1, 0.5:1, 0.25:1) using a paired t test. There were no statistically significant results from control group to decellularized group for any of the loading ratios (Figure 5-6).

Figure 5-6. Circumferential low-tension modulus post-treatment values from the control group are compared to the post-treatment values from the decellularized group with a paired t test. Statistical significance is indicated with $* = p < 0.05$, and $** = p < 0.005$.

The circumferential low-tension modulus values were averaged and the percent change calculated between the pre-treatment and post-treatment values (Tables 5-2 and 5-3).

Table 5-2. Circumferential low-tension modulus values and % change from pre-treatment to post-treatment for the control group.

Control								
	Tissue 1	Tissue 2	Tissue 3	Tissue 4	Tissue 5	AVG	SEM	% Change
1:1, Pre-Treatment	4.10902	1.083965	2.055272	2.320227	2.383383	2.39037349	0.4887592	-19%
1:1, Post-Treatment	1.045986	2.295636	-4.880797	3.344397	7.876598	1.93636383	2.05747984	
1:0.75, Pre-Treatment	4.29306	0.795558	1.9528	2.92972	0.879645	2.17015655	0.65900841	92%
1:0.75, Post-Treatment	2.234037	1.906193	6.604673	4.017294	6.124316	4.17730266	0.96547432	
1:0.50, Pre-Treatment	3.793949	1.277352	2.359854	2.192533	4.799337	2.884605	0.62572724	7%
1:0.50, Post-Treatment	2.423478	3.125149	2.868005	3.86635	3.199697	3.09653573	0.23551094	
1:0.25, Pre-Treatment	3.814634	0.883937	1.011189	2.677745	1.364653	1.95043175	0.56422338	40%
1:0.25, Post-Treatment	2.327755	2.471036	1.722098	3.139477	4.026846	2.73744218	0.39331492	
0.75:1, Pre-Treatment	5.236472	1.587886	1.240901	2.747819	7.492967	3.6612089	1.18647943	-3%
0.75:1, Post-Treatment	2.273574	0.382021	6.875435	3.342338	4.95954	3.56658178	1.1124843	
0.50:1, Pre-Treatment	3.243477	0.810718	1.773563	3.122644	5.712538	2.93258812	0.82784553	-27%
0.50:1, Post-Treatment	2.3203	2.625157	-0.495502	3.226182	3.076815	2.15059055	0.68083063	
0.25:1, Pre-Treatment	6.171801	0.410717	4.813986	2.737635	6.048603	4.03654822	1.09649232	-6%
0.25:1, Post-Treatment	1.303817	8.231226	-0.475985	4.042148	5.861461	3.79253327	1.55657838	

Table 5-3. Circumferential low-tension modulus values and % change from pre-treatment to post-treatment for the decellularized group.

Decellularized								
	Tissue 1	Tissue 2	Tissue 3	Tissue 4	Tissue 5	AVG	SEM	% Change
1:1, Pre-Treatment	2.360227	21.93409	21.59979	4.657377	3.06517	10.7233314	4.52417538	-71%
1:1, Post-Treatment	7.901501	1.448877	2.101227	2.212084	1.979105	3.1285586	1.20039347	
1:0.75, Pre-Treatment	2.791896	23.49269	18.85616	3.949049	-6.783163	8.46132727	5.56278755	-69%
1:0.75, Post-Treatment	5.570401	4.130886	1.982082	0.999115	0.544194	2.64533562	0.95727776	
1:0.50, Pre-Treatment	2.964883	25.8032	15.10627	4.376057	4.99433	10.6489474	4.3582549	-77%
1:0.50, Post-Treatment	6.8552	1.324792	1.464577	1.841201	0.830711	2.46329633	1.10983755	
1:0.25, Pre-Treatment	2.456967	20.87125	10.26306	2.574509	3.826433	7.99844439	3.52399187	-72%
1:0.25, Post-Treatment	6.073611	0.393309	1.282694	1.484858	1.765479	2.19999018	0.99525577	
0.75:1, Pre-Treatment	3.143012	17.85563	19.19191	3.707836	1.650927	9.10986254	3.86365741	-78%
0.75:1, Post-Treatment	6.04739	0.949821	1.644706	2.923546	-1.599556	1.99318145	1.25339513	
0.50:1, Pre-Treatment	3.433077	15.44756	18.24141	2.35451	4.612984	8.81790757	3.32571757	-67%
0.50:1, Post-Treatment	6.584361	1.310973	2.453584	3.533277	0.864518	2.9493426	1.02056021	
0.25:1, Pre-Treatment	3.10537	16.78784	67.24731	10.32748	9.38431	21.3704595	11.6724342	-88%
0.25:1, Post-Treatment	5.49475	0.893289	2.393125	3.566916	0.341549	2.53792591	0.93143698	

For each of the seven loading ratios (T_{circ}:T_{rad} = 1:1, 1:0.75, 1:0.5, 1:0.25, 0.75:1, 0.5:1, 0.25:1), the pre-treatment value and the post-treatment value for the low tension modulus in the radial direction were compared to the using a paired t test. No statistically significant results were found from pre-treatment to post-treatment for any of the loading ratios, for either the control group or the decellularized group.
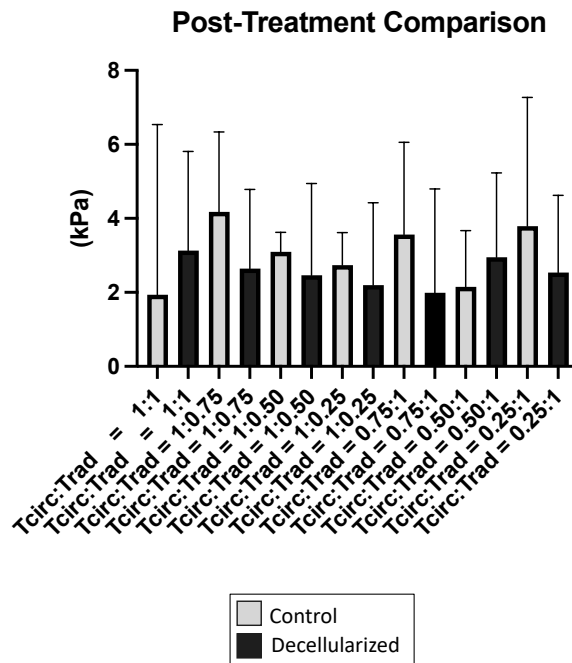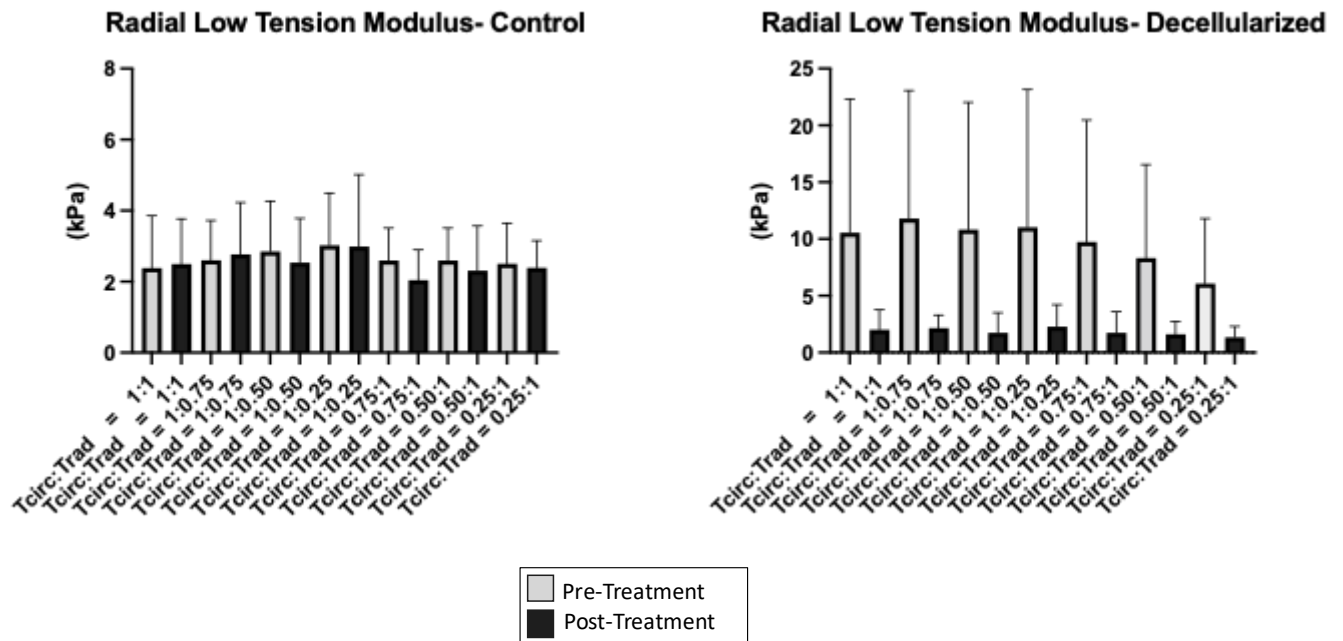

Figure 5-7. Radial low-tension modulus for control and decellularized treatment groups. Pre-treatment values are compared to post-treatment values with a paired t test. Statistical significance is indicated with $* = p < 0.05$, and $** = p < 0.005$.

Additionally for the low-tension modulus in the radial direction, the post-treatment value for the control group was compared to the post-treatment value for the decellularized group for each loading protocol (T_{circ}:T_{rad} = 1:1, 1:0.75, 1:0.5, 1:0.25, 0.75:1, 0.5:1, 0.25:1) using a paired t test. There were no statistically significant results from control group to decellularized group for any of the loading ratios (Figure 5-8).

Figure 5-8. Radial low-tension modulus post-treatment values from the control group are compared to the post-treatment values from the decellularized group with a paired t test. Statistical significance is indicated with $* = p < 0.05$, and $** = p < 0.005$.

The radial low-tension modulus values were averaged and the percent change calculated between the pre-treatment and post-treatment values (Tables 5-4 and 5-5).

Table 5-4. Radial low-tension modulus values and % change from pre-treatment to post-treatment for the control group.

Control								
	Tissue 1	Tissue 2	Tissue 3	Tissue 4	Tissue 5	AVG	SEM	% Change
1:1, Pre-Treatment	3.50633	1.442641	0.70754	4.27581	1.970618	2.38058766	0.65977533	5%
1:1, Post-Treatment	2.44651	1.323019	4.44708	2.864702	1.398009	2.49586387	0.57125175	
1:0.75, Pre-Treatment	3.598895	1.434463	1.434904	3.730244	2.804684	2.60063765	0.50165032	6%
1:0.75, Post-Treatment	2.287847	1.561701	5.128825	3.15527	1.703689	2.76746651	0.65352645	
1:0.50, Pre-Treatment	3.543925	2.199993	0.719156	4.410121	3.360227	2.84668438	0.63791816	-11%
1:0.50, Post-Treatment	1.97266	2.293691	4.440287	2.883935	1.108571	2.53982866	0.55501874	
1:0.25, Pre-Treatment	3.410426	2.032044	1.3429	5.187901	3.14353	3.02336019	0.6578811	-1%
1:0.25, Post-Treatment	1.973418	1.100605	6.303608	3.411096	2.146681	2.9870818	0.9075162	
0.75:1, Pre-Treatment	3.435117	1.862695	1.568189	3.61238	2.51856	2.5993882	0.40848598	-21%
0.75:1, Post-Treatment	2.023383	0.885922	3.258331	2.219369	1.83879	2.04515871	0.38006764	
0.50:1, Pre-Treatment	3.304025	1.584442	1.658696	2.993139	3.455614	2.59918315	0.40618307	-11%
0.50:1, Post-Treatment	1.718046	1.372318	4.25896	2.885809	1.304997	2.30802613	0.56434341	
0.25:1, Pre-Treatment	3.55108	1.312265	1.218272	3.464652	2.955339	2.5003216	0.51459681	-4%
0.25:1, Post-Treatment	1.709833	2.387684	2.89914	3.378687	1.569117	2.38889225	0.34447053	

Table 5-5. Radial low-tension modulus values and % change from pre-treatment to post-treatment for the decellularized group.

Decellularization								
	Tissue 1	Tissue 2	Tissue 3	Tissue 4	Tissue 5	AVG	SEM	% Change
1:1, Pre-Treatment	1.410569	17.72023	27.89563	2.946576	2.788101	10.552221	4.29678673	-81%
1:1, Post-Treatment	4.243025	0.749647	3.420299	0.175165	1.503526	2.01833209	0.63735452	
1:0.75, Pre-Treatment	2.254756	19.23493	27.88045	3.008616	6.61751	11.7992524	4.11953645	-82%
1:0.75, Post-Treatment	3.758942	1.702527	2.916314	0.983693	1.389141	2.15012319	0.4209252	
1:0.50, Pre-Treatment	2.711793	19.4545	26.14869	2.17238	3.636329	10.8247385	4.08922457	-84%
1:0.50, Post-Treatment	4.045305	0.12529	3.252858	0.657387	0.627973	1.74176249	0.6485651	
1:0.25, Pre-Treatment	2.969487	15.65505	30.38088	2.153999	3.984998	11.0288822	4.43183506	-79%
1:0.25, Post-Treatment	4.254072	0.392017	4.497903	1.357452	0.939149	2.28811867	0.70779428	
0.75:1, Pre-Treatment	1.971654	16.57457	25.37797	1.937486	2.851675	9.74267257	3.91549509	-82%
0.75:1, Post-Treatment	3.626976	-0.406965	3.753349	0.924049	0.844606	1.74840284	0.6755459	
0.50:1, Pre-Treatment	2.441858	13.83865	20.13007	2.065923	3.087699	8.31283837	3.00541666	-81%
0.50:1, Post-Treatment	3.049413	0.830298	2.641225	0.676077	0.838134	1.60702963	0.41678198	
0.25:1, Pre-Treatment	1.692749	11.49924	13.10613	1.374271	2.676268	6.06973187	2.09532339	-78%
0.25:1, Post-Treatment	2.644637	1.027387	2.019354	0.291877	0.693012	1.3352533	0.35488818	

The same data analysis was performed for the high-tension modulus. For each of the seven loading ratios (T_{circ}:T_{rad} = 1:1, 1:0.75, 1:0.5, 1:0.25, 0.75:1, 0.5:1, 0.25:1), the pre-treatment value and the post-treatment value for the high-tension modulus in the circumferential direction were compared to the using a paired t test. No statistically significant results were found from pre-treatment to post-treatment for any of the loading ratios, for either the control group or the decellularized group.
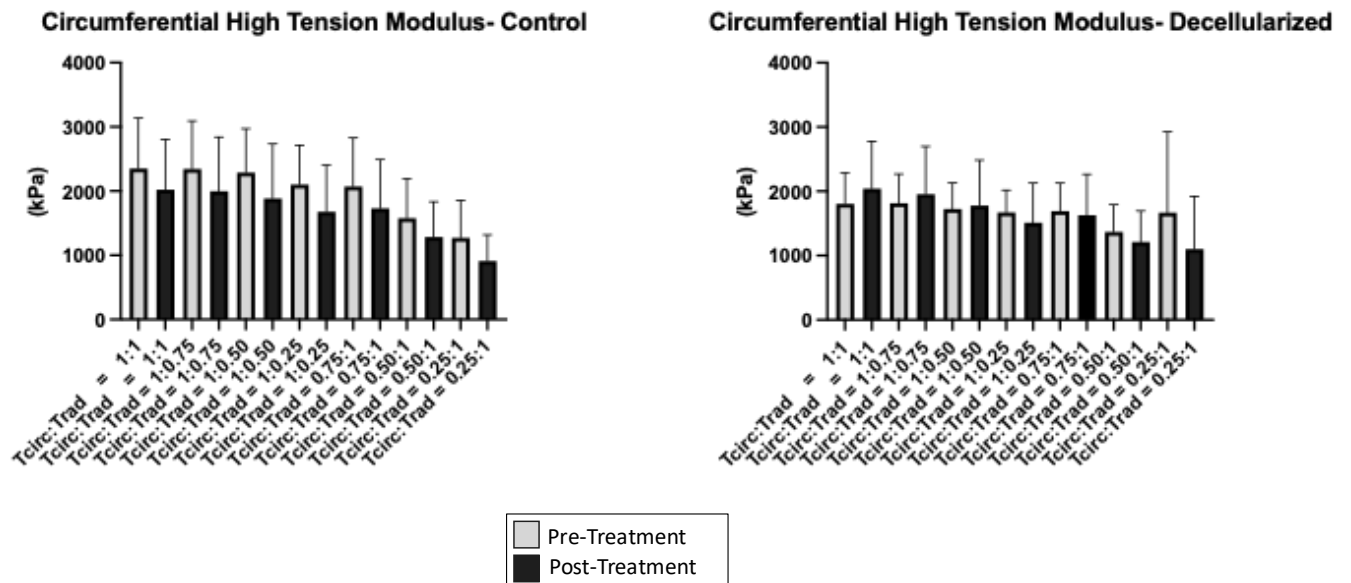
Figure 5-9. Circumferential high-tension modulus for control and decellularized treatment groups. Pre-treatment values are compared to post-treatment values with a paired t test. Statistical significance is indicated with $* = p < 0.05$, and $** = p < 0.005$.

For the high-tension modulus in the circumferential direction, the post-treatment value for the control group was compared to the post-treatment value for the decellularized group for each loading protocol (T_{circ}:T_{rad} = 1:1, 1:0.75, 1:0.5, 1:0.25, 0.75:1, 0.5:1, 0.25:1) using a paired t test. There were no statistically significant results from control group to decellularized group for any of the loading ratios (Figure 5-10).

Figure 5-10. Circumferential high-tension modulus post-treatment values from the control group are compared to the post-treatment values from the decellularized group with a paired t test. Statistical significance is indicated with $* = p < 0.05$, and $** = p < 0.005$.

The circumferential high-tension modulus values were averaged and the percent change calculated between the pre-treatment and post-treatment values (Tables 5-6 and 5-7).

Table 5-6. Circumferential high-tension modulus values and % change from pre-treatment to post-treatment for the control group.

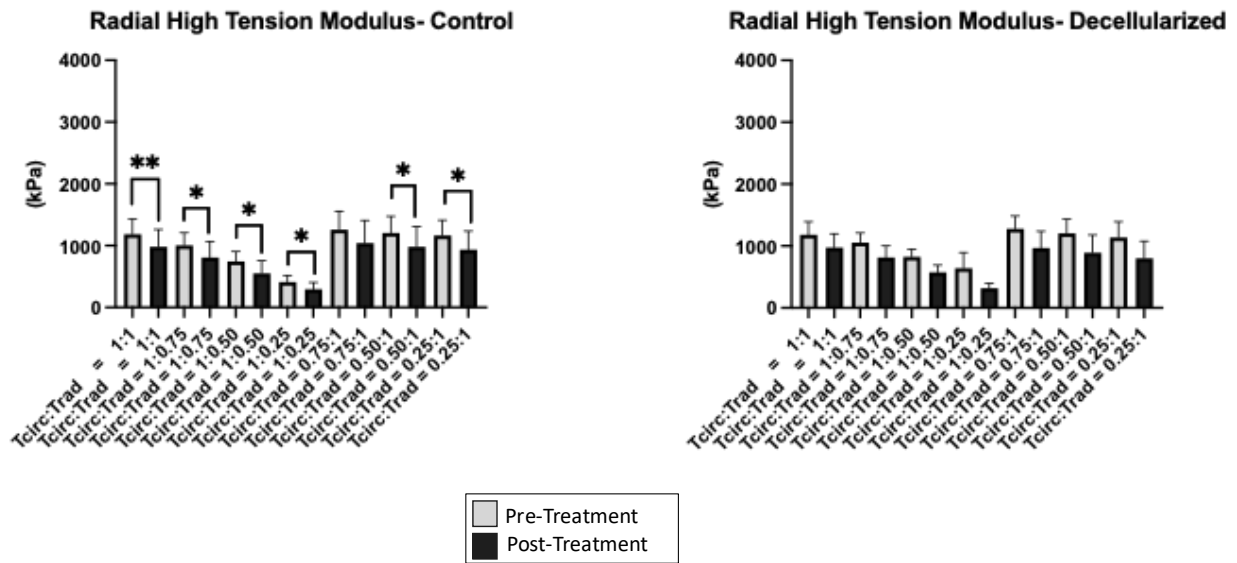| Control								
	Tissue 1	Tissue 2	Tissue 3	Tissue 4	Tissue 5	AVG	SEM	% Change
1:1, Pre-Treatment	1600.505	2034.399	3535.492	1835.955	2748.365	2350.94339	352.822135	-14%
1:1, Post-Treatment	650.2002	2113.654	2407.523	2355.739	2581.264	2021.67605	350.928808	
1:0.75, Pre-Treatment	1563.936	2035.328	3319.377	1872.964	2931.832	2344.6876	333.358529	-15%
1:0.75, Post-Treatment	521.5154	2087.959	2434.523	2437.4	2510.736	1998.42676	376.471903	
1:0.50, Pre-Treatment	1505.511	1989.414	3036.578	1935.224	2976.1	2288.56566	304.937892	-18%
1:0.50, Post-Treatment	392.299	1978.662	2330.672	2266.184	2453.731	1884.30954	381.07126	
1:0.25, Pre-Treatment	1346.736	1825.068	2701.777	1871.68	2768.177	2102.68769	274.197313	-20%
1:0.25, Post-Treatment	395.5036	1819.791	2021.42	2105.479	2052.007	1678.84017	324.457621	
0.75:1, Pre-Treatment	1349.103	1655.018	3143.416	1643.934	2571.982	2072.69054	337.461493	-17%
0.75:1, Post-Treatment	418.8434	1713.397	2247.735	2111.542	2160.071	1730.31778	340.440287	
0.50:1, Pre-Treatment	1029.513	1241.275	2471.244	1175.96	1970.681	1577.73479	276.400251	-18%
0.50:1, Post-Treatment	350.5541	1243.501	1649.023	1523.226	1663.416	1285.94404	245.699987	
0.25:1, Pre-Treatment	794.1646	1172.822	2069.343	672.2816	1645.269	1270.77605	262.002186	-28%
0.25:1, Post-Treatment	243.4143	1156.421	1019.139	856.9835	1285.487	912.289142	181.761753	

Table 5-7. Circumferential high-tension modulus values and % change from pre-treatment to post-treatment for the decellularized group.

Decellularized								
	Tissue 1	Tissue 2	Tissue 3	Tissue 4	Tissue 5	AVG	SEM	% Change
1:1, Pre-Treatment	1769.363	1246.673	1498.588	1997.276	2509.263	1804.23266	216.776543	
1:1, Post-Treatment	888.9707	2127.876	1885.764	2823.8	2487.147	2042.71132	329.440816	13%
1:0.75, Pre-Treatment	1716.604	1292.306	1597.968	1978.436	2495.594	1816.18144	202.473022	
1:0.75, Post-Treatment	791.1612	2111.71	1760.809	2767.403	2338.762	1953.96876	333.470746	8%
1:0.50, Pre-Treatment	1680.137	1244.895	1455.98	1968.993	2271.259	1724.25277	182.045917	
1:0.50, Post-Treatment	679.0772	1839.644	1666.153	2577.533	2131.022	1778.68603	315.26307	3%
1:0.25, Pre-Treatment	1503.708	1253.453	1531.901	2083.798	1978.651	1670.30213	156.001616	
1:0.25, Post-Treatment	532.8434	1595.211	1449.733	2200.396	1779.024	1511.44148	275.203419	-10%
0.75:1, Pre-Treatment	1618.264	1227.537	1524.879	1684.496	2404.49	1691.93318	194.52385	
0.75:1, Post-Treatment	651.4137	1662.46	1458.085	2306.314	2056.521	1626.95867	285.264238	-4%
0.50:1, Pre-Treatment	1208.333	937.6606	1322.65	1292.699	2077.317	1367.73192	189.939883	
0.50:1, Post-Treatment	463.6651	1201.207	1112.941	1655.47	1621.345	1210.92578	216.120048	-11%
0.25:1, Pre-Treatment	792.8888	856.2458	3789.622	1074.918	1830.887	1668.91239	561.440928	
0.25:1, Post-Treatment	277.736	714.9134	993.682	2455.843	1069.68	1102.37074	365.712573	-34%

Finally, for each of the seven loading ratios (T_{circ}:T_{rad} = 1:1, 1:0.75, 1:0.5, 1:0.25, 0.75:1, 0.5:1, 0.25:1), the pre-treatment value and the post-treatment value for the high-tension modulus in the radial direction were compared to the using a paired t test. No statistically significant results were found from pre-treatment to post-treatment for any of the loading ratios for the decellularized group. However, six out of seven loading ratios showed statistically significant results between pre-treatment and post-treatment values for the control group.

Figure 5-11. Radial high-tension modulus for control and decellularized treatment groups. Pre-treatment values are compared to post-treatment values with a paired t test. Statistical significance is indicated with $* = p < 0.05$, and $** = p < 0.005$.

For the high-tension modulus in the radial direction, the post-treatment value for the control group was compared to the post-treatment value for the decellularized group for each loading protocol ($T_{circ}:T_{rad}$ = 1:1, 1:0.75, 1:0.5, 1:0.25, 0.75:1, 0.5:1, 0.25:1) using a paired t test. There were no statistically significant results from control group to decellularized group for any of the loading ratios (Figure 5-12).

Figure 5-12. Radial high-tension modulus post-treatment values from the control group are compared to the post-treatment values from the decellularized group with a paired t test. Statistical significance is indicated with $* = p < 0.05$, and $** = p < 0.005$.

The radial high-tension modulus values were averaged and the percent change calculated between the pre-treatment and post-treatment values (Tables 5-8 and 5-9).

Table 5-8. Radial high-tension modulus values and % change from pre-treatment to post-treatment for the control group.

| Control								
	Tissue 1	Tissue 2	Tissue 3	Tissue 4	Tissue 5	AVG	SEM	% Change
1:1, Pre-Treatment	855.1337	1176.131	1192.485	1552.92	1146.278	1184.58951	110.862508	-17%
1:1, Post-Treatment	560.946	1111.045	1019.167	1319.224	891.4622	980.368966	125.941295	
1:0.75, Pre-Treatment	718.0962	1011.453	957.055	1278.618	1065.605	1006.16544	90.3291268	-20%
1:0.75, Post-Treatment	400.0375	909.9954	901.6372	1094.778	728.5696	807.003452	117.079016	
1:0.50, Pre-Treatment	516.7302	777.2989	667.9016	933.4453	834.3693	745.949056	71.6199917	-26%
1:0.50, Post-Treatment	209.3183	616.8801	655.2747	733.0434	546.2275	552.14878	90.861369	
1:0.25, Pre-Treatment	252.2736	424.2343	380.2116	542.5714	461.6754	412.193251	48.056755	-29%
1:0.25, Post-Treatment	113.6512	327.3266	355.0693	394.1745	269.2279	291.889886	48.9857741	
0.75:1, Pre-Treatment	842.7362	1186.164	1251.376	1676.555	1331.439	1257.65391	133.833715	-17%
0.75:1, Post-Treatment	486.11	1168.062	1181.123	1460.525	908.8318	1040.93036	163.882817	
0.50:1, Pre-Treatment	818.1853	1130.566	1241.825	1559.473	1281.543	1206.31846	120.004951	-19%
0.50:1, Post-Treatment	514.2083	1045.008	1085.601	1402.348	864.5492	982.342947	145.605451	
0.25:1, Pre-Treatment	813.839	1076.081	1143.039	1440.322	1351.87	1165.03014	110.0967	-20%
0.25:1, Post-Treatment	538.5089	929.8122	987.4302	1375.006	840.103	934.172091	134.645532	

Table 5-9. Radial high-tension modulus values and % change from pre-treatment to post-treatment for the decellularized group.

Decellularized								
	Tissue 1	Tissue 2	Tissue 3	Tissue 4	Tissue 5	AVG	SEM	% Change
1:1, Pre-Treatment	1471.196	877.6081	1194.253	1207.869	1150.549	1180.295	94.361709	
1:1, Post-Treatment	749.6117	1285.2	863.777	856.1671	1125.904	976.131895	99.0659223	-17%
1:0.75, Pre-Treatment	1224.634	816.425	1163.104	1044.896	1022.176	1054.24715	70.198587	
1:0.75, Post-Treatment	575.0392	1065.746	741.4478	764.7546	920.3737	813.47224	83.5108343	-23%
1:0.50, Pre-Treatment	943.3988	649.7565	929.7543	842.9613	757.6657	824.707311	55.0264047	
1:0.50, Post-Treatment	392.6014	689.8592	563.558	575.803	660.9619	576.5567	51.9474716	-30%
1:0.25, Pre-Treatment	559.4971	458.1954	1079.306	621.5601	497.0695	643.125578	112.526328	
1:0.25, Post-Treatment	178.578	368.8402	308.9111	360.7661	370.7191	317.562923	36.546617	-51%
0.75:1, Pre-Treatment	1564.972	1004.348	1323.636	1185.72	1317.725	1279.28003	92.0344102	
0.75:1, Post-Treatment	660.5202	1300.875	815.3935	859.8133	1202.037	967.727755	121.473349	-24%
0.50:1, Pre-Treatment	1503.242	902.6144	1206.994	1074.975	1332.146	1203.99421	103.309461	
0.50:1, Post-Treatment	601.1158	1213.981	750.0476	713.0022	1183.154	892.260162	127.523834	-26%
0.25:1, Pre-Treatment	1490.583	908.5355	1043.923	937.8293	1309.341	1138.04233	112.97012	
0.25:1, Post-Treatment	527.1352	1079.91	607.361	686.1639	1114.006	802.915245	122.766156	-29%

The peak tissue stretch, an approximate measure of overall tissue extensibility, was measured for each loading protocol in the circumferential and radial directions. This value was compared before and after treatment with a paired *t* test for both the control and decellularized groups.

In the circumferential direction, there is a general trend of higher extensibility post-treatment as compared to pre-treatment. However, these results are observed in both the control tissues as well as the decellularized tissues, indicating that this observation is most likely a result of the tissues soaking in solution for a week, rather than the decellularization treatment itself (Figure 5-13).
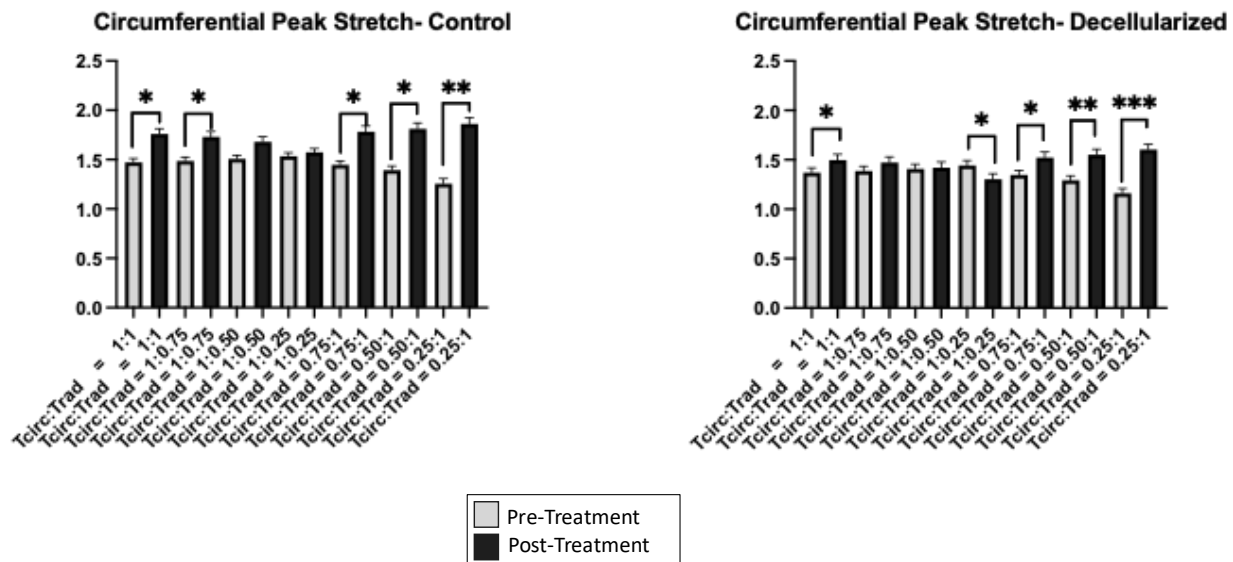
Figure 5-13. Peak stretch values for each loading protocol before treatment and after treatment in the circumferential direction. Statistical significance is indicated with $* = p < 0.05$, and $** = p < 0.005$.

For the peak stretch in the circumferential direction, the post-treatment value for the control group was compared to the post-treatment value for the decellularized group for each loading protocol (T_{circ}:T_{rad} = 1:1, 1:0.75, 1:0.5, 1:0.25, 0.75:1, 0.5:1, 0.25:1) using a paired t test. Out of the seven loading ratios, two showed statistically significant results, with a decrease in the decellularized group as compared to the control group (Figure 5-14).
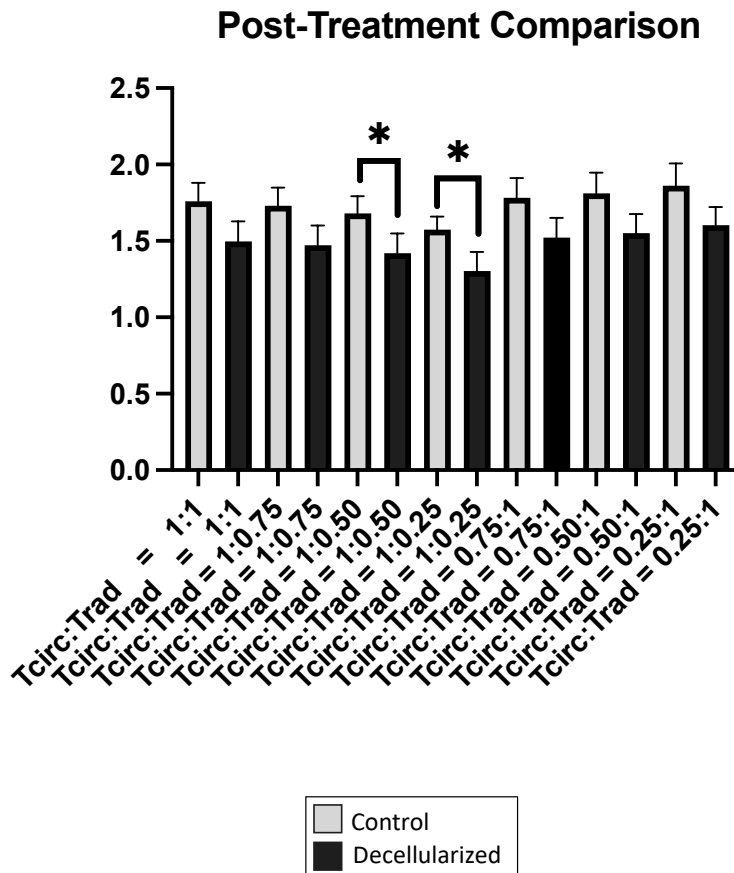
Figure 5-14. Circumferential peak stretch post-treatment values from the control group are compared to the post-treatment values from the decellularized group with a paired t test. Statistical significance is indicated with $* = p < 0.05$, and $** = p < 0.005$.

The circumferential peak stretch values were averaged and the percent change calculated between the pre-treatment and post-treatment values (Tables 5-10 and 5-11).

Table 5-10. Circumferential peak stretch values and % change from pre-treatment to post-treatment for the control group.

| Control								
	Tissue 1	Tissue 2	Tissue 3	Tissue 4	Tissue 5	AVG	SEM	% Change
1:1, Pre-Treatment	1.4972	1.526197	1.347954	1.568591	1.423067	1.47260153	0.03916776	
1:1, Post-Treatment	1.812958	1.7676	1.893081	1.560533	1.757899	1.7584143	0.05493253	19%
1:0.75, Pre-Treatment	1.514133	1.545127	1.362352	1.578056	1.435333	1.48700039	0.03912345	
1:0.75, Post-Treatment	1.781362	1.7344	1.862952	1.54	1.735902	1.73092319	0.05313387	16%
1:0.50, Pre-Treatment	1.537333	1.569657	1.383416	1.589788	1.452267	1.50649219	0.03870884	
1:0.50, Post-Treatment	1.72657	1.679067	1.811625	1.502667	1.683909	1.68076741	0.05047486	12%
1:0.25, Pre-Treatment	1.5624	1.60312	1.421944	1.604986	1.4756	1.53360987	0.03646196	
1:0.25, Post-Treatment	1.634849	1.564533	1.650847	1.4324	1.584722	1.57347012	0.03864045	3%
0.75:1, Pre-Treatment	1.469333	1.488202	1.323557	1.553793	1.396933	1.44636359	0.03958782	
0.75:1, Post-Treatment	1.833089	1.7996	1.917211	1.571867	1.791628	1.78267888	0.05720501	23%
0.50:1, Pre-Treatment	1.409467	1.411812	1.273164	1.525397	1.348	1.39356772	0.04155602	
0.50:1, Post-Treatment	1.867084	1.836667	1.948407	1.586667	1.820157	1.81179638	0.06045288	30%
0.25:1, Pre-Treatment	1.25	1.194374	1.156779	1.455006	1.218267	1.25488517	0.052297	
0.25:1, Post-Treatment	1.923077	1.899467	1.9972	1.615867	1.874817	1.86208546	0.06486876	48%

Table 5-11. Circumferential peak stretch values and % change from pre-treatment to post-treatment for the decellularized group.

Decellularized								
	Tissue 1	Tissue 2	Tissue 3	Tissue 4	Tissue 5	AVG	SEM	% Change
1:1, Pre-Treatment	1.526996	1.336533	1.272533	1.420933	1.294894	1.37037808	0.04663799	
1:1, Post-Treatment	1.622317	1.433809	1.296627	1.5724	1.554993	1.49602913	0.05869002	9%
1:0.75, Pre-Treatment	1.541528	1.3528	1.290133	1.4408	1.308492	1.38675067	0.04663112	
1:0.75, Post-Treatment	1.59832	1.413145	1.271697	1.536533	1.536862	1.47131147	0.05827143	6%
1:0.50, Pre-Treatment	1.565391	1.3708	1.3088	1.465467	1.327956	1.40768284	0.0478045	
1:0.50, Post-Treatment	1.556193	1.369551	1.222637	1.468267	1.485802	1.42048976	0.05775121	1%
1:0.25, Pre-Treatment	1.614985	1.401333	1.340667	1.4944	1.361818	1.44264062	0.05050679	
1:0.25, Post-Treatment	1.411012	1.282762	1.09772	1.357067	1.368884	1.30348906	0.05544304	-10%
0.75:1, Pre-Treatment	1.507266	1.315467	1.254	1.388133	1.268631	1.34669931	0.04645518	
0.75:1, Post-Treatment	1.639515	1.461272	1.324223	1.600133	1.582322	1.52149314	0.05760014	13%
0.50:1, Pre-Treatment	1.455539	1.267333	1.192	1.319467	1.22717	1.29230179	0.04599482	
0.50:1, Post-Treatment	1.665645	1.489935	1.363152	1.635733	1.604586	1.55181004	0.05577065	20%
0.25:1, Pre-Treatment	1.32609	1.156667	1.029733	1.160267	1.132116	1.16097445	0.04761261	
0.25:1, Post-Treatment	1.713905	1.534329	1.432742	1.694533	1.642581	1.60361804	0.0528897	38%

The same general trend of higher extensibility post-treatment as compared to pre-treatment was observed in the radial direction as well. Once again, these results are observed in both the control tissues as well as the decellularized tissues, indicating that this observation is most likely a result of the tissues soaking in solution for a week, rather than the decellularization treatment itself (Figure 5-15).
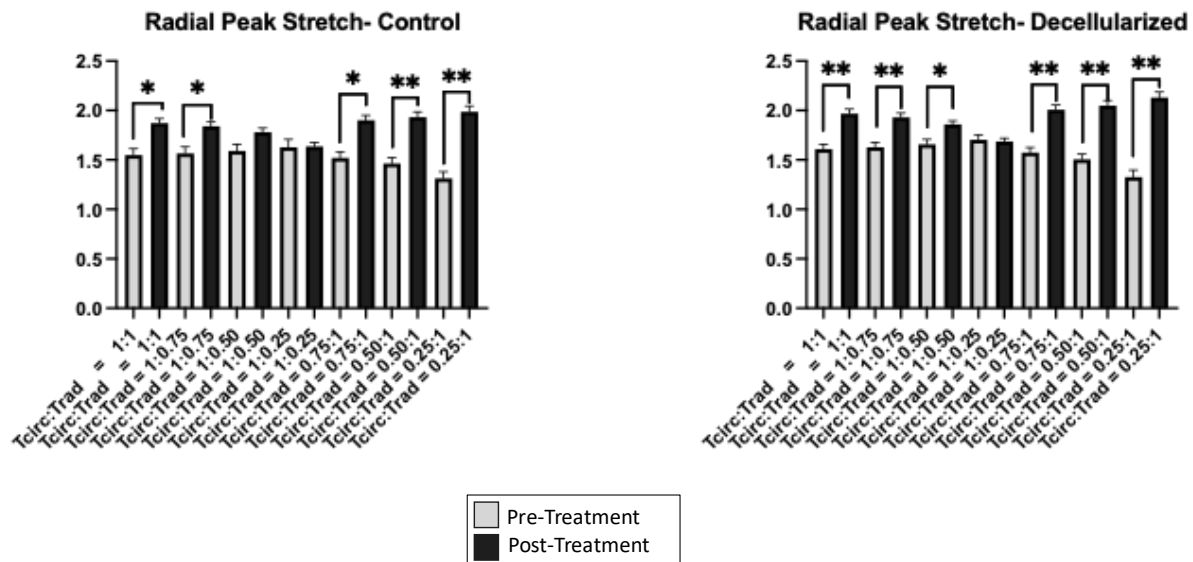
Figure 5-15. Peak stretch values for each loading protocol before treatment and after treatment in the radial direction. Statistical significance is indicated with $* = p < 0.05$, and $** = p < 0.005$.

For the peak stretch in the radial direction, the post-treatment value for the control group was compared to the post-treatment value for the decellularized group for each loading protocol ($T_{circ}:T_{rad}$ = 1:1, 1:0.75, 1:0.5, 1:0.25, 0.75:1, 0.5:1, 0.25:1) using a paired t test. None of the loading ratios showed statistically significant results (Figure 5-14).

Post-Treatment Comparison
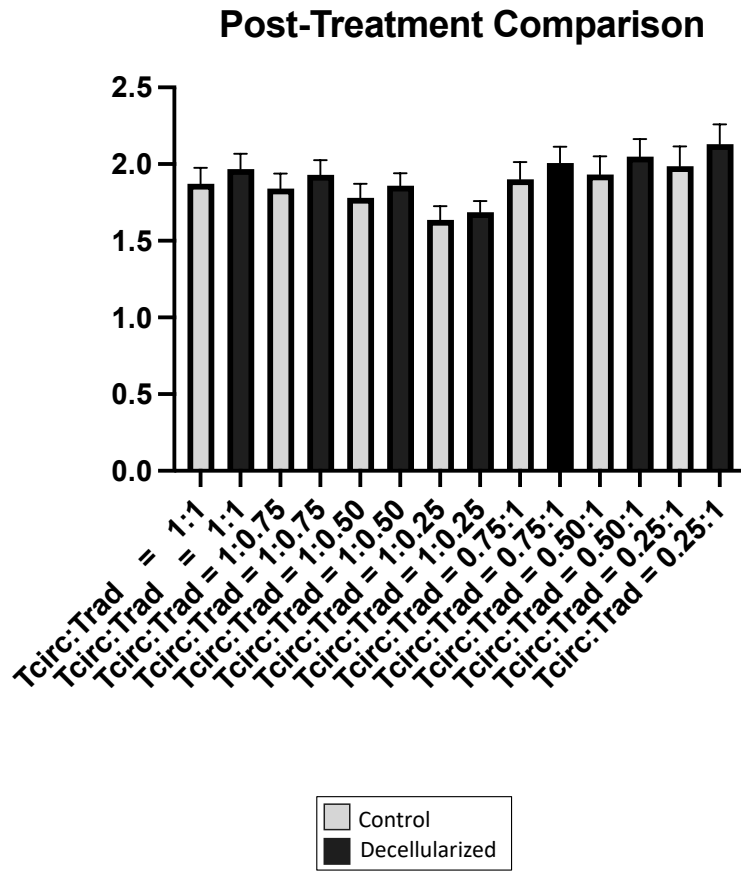
Figure 5-16. Radial peak stretch post-treatment values from the control group are compared to the post-treatment values from the decellularized group with a paired t test. Statistical significance is indicated with $* = p < 0.05$, and $** = p < 0.005$.

The radial peak stretch values were averaged and the percent change calculated between the pre-treatment and post-treatment values (Tables 5-12 and 5-13).

Table 5-12. Radial peak stretch values and % change from pre-treatment to post-treatment for the control group.

	Tissue 1	Tissue 2	Tissue 3	Tissue 4	Tissue 5	AVG	SEM	% Change
Control								
1:1, Pre-Treatment	1.799385	1.532995	1.46639	1.515538	1.433077	1.54947694	0.06493449	
1:1, Post-Treatment	1.8994	1.864154	1.814308	1.749885	2.030611	1.87167139	0.04701579	21%
1:0.75, Pre-Treatment	1.822308	1.549608	1.480541	1.524	1.454615	1.56621446	0.06611951	
1:0.75, Post-Treatment	1.860483	1.831231	1.790769	1.727427	1.991386	1.8402591	0.04388067	17%
1:0.50, Pre-Treatment	1.863077	1.572681	1.502692	1.535077	1.476154	1.58993614	0.07016806	
1:0.50, Post-Treatment	1.784187	1.775385	1.726923	1.688202	1.927703	1.78048	0.04068049	12%
1:0.25, Pre-Treatment	1.939077	1.607906	1.534995	1.550923	1.504462	1.62747253	0.07969436	
1:0.25, Post-Treatment	1.57868	1.634923	1.566308	1.611137	1.789878	1.63618524	0.04027427	1%
0.75:1, Pre-Treatment	1.761077	1.494385	1.438394	1.499231	1.403231	1.51926361	0.06304658	
0.75:1, Post-Treatment	1.932164	1.895692	1.850615	1.762344	2.06722	1.90160733	0.05019502	25%
0.50:1, Pre-Treatment	1.691077	1.422089	1.391324	1.471231	1.344154	1.46397497	0.06042251	
0.50:1, Post-Treatment	1.974004	1.930154	1.882308	1.775573	2.099369	1.93228157	0.05325344	32%
0.25:1, Pre-Treatment	1.539231	1.195662	1.265805	1.390154	1.173385	1.31284734	0.06805179	
0.25:1, Post-Treatment	2.029534	2.003077	1.943385	1.803415	2.154745	1.98683115	0.05736366	51%

Table 5-13. Radial peak stretch values and % change from pre-treatment to post-treatment for the decellularized group.

	Tissue 1	Tissue 2	Tissue 3	Tissue 4	Tissue 5	AVG	SEM	% Change
Decellularized								
1:1, Pre-Treatment	1.733579	1.631749	1.621443	1.426242	1.632672	1.60913706	0.05007649	
1:1, Post-Treatment	1.929088	1.836487	2.03261	1.948	2.095231	1.96828313	0.04448127	22%
1:0.75, Pre-Treatment	1.758191	1.645593	1.64267	1.44747	1.650054	1.62879557	0.05027368	
1:0.75, Post-Treatment	1.898016	1.808952	1.986464	1.9	2.058462	1.93037866	0.04258244	19%
1:0.50, Pre-Treatment	1.786802	1.663129	1.675435	1.481157	1.683126	1.65792955	0.04940903	
1:0.50, Post-Treatment	1.848331	1.76219	1.903861	1.808308	1.970769	1.85869187	0.03644175	12%
1:0.25, Pre-Treatment	1.839255	1.695893	1.720966	1.526842	1.734502	1.70349177	0.05047855	
1:0.25, Post-Treatment	1.736656	1.62252	1.727734	1.589692	1.751846	1.68568963	0.03312697	-1%
0.75:1, Pre-Treatment	1.706199	1.609599	1.570835	1.383787	1.595754	1.57323489	0.05263323	
0.75:1, Post-Treatment	1.951238	1.858329	2.083526	2.007231	2.131077	2.00628021	0.04819803	28%
0.50:1, Pre-Treatment	1.633287	1.559914	1.477311	1.315028	1.53884	1.50487617	0.05360694	
0.50:1, Post-Treatment	1.989079	1.889709	2.143363	2.062615	2.165231	2.04999932	0.05077321	36%
0.25:1, Pre-Treatment	1.485771	1.421935	1.214429	1.106138	1.401784	1.32601138	0.07115526	
0.25:1, Post-Treatment	2.034918	1.954622	2.258268	2.167231	2.231077	2.12922314	0.05824529	61%

A one-way multivariate analysis of variance (MANOVA) was used to determine if there are any differences between decellularized and control groups for six dependent variables (circumferential high tension modulus, radial high tension modulus, circumferential low tension modulus, radial low tension modulus, circumferential peak stretch, and radial peak stretch). In this study, the independent variable is treatment, which has two groups of decellularized and control. This one-way MANOVA test was repeated for each loading ratio (T_{circ}:T_{rad} = 1:1, 1:0.75, 1:0.5, 1:0.25, 0.75:1, 0.5:1, 0.25:1). There were no statistically significant results for any of the loading ratios, so no post-hoc analysis was attempted (Table 5-14).

Table 5-14. *P*-value results from one-way MANOVA repeated for each loading protocol.

Tcirc:Trad	P Value	Significance
1:1	0.54	n.s.
1:0.75	0.56	n.s.
1:0.5	0.52	n.s.
1:0.25	0.44	n.s.
0.75:1	0.42	n.s.
0.5:1	0.53	n.s.
0.25:1	0.35	n.s.

5.3 Results- pSFDI Analysis

pSFDI analysis was performed with the tissue in four biaxial loading ratios (T_{circ}:T_{rad} = mounting, 1:1, 1:0.25, 0.25:1). In order to determine if the decellularization treatment influences average θ_{Fiber} across the leaflet, pre-treatment θ Fiber values were compared to post-treatment θ_{Fiber} values for each loading protocol for both the control and decellularized groups using a paired t test. No statistically significant results were found from pre-treatment to post-treatment for any of the loading ratios, in either the control group or the decellularized group (Figure 5-17).
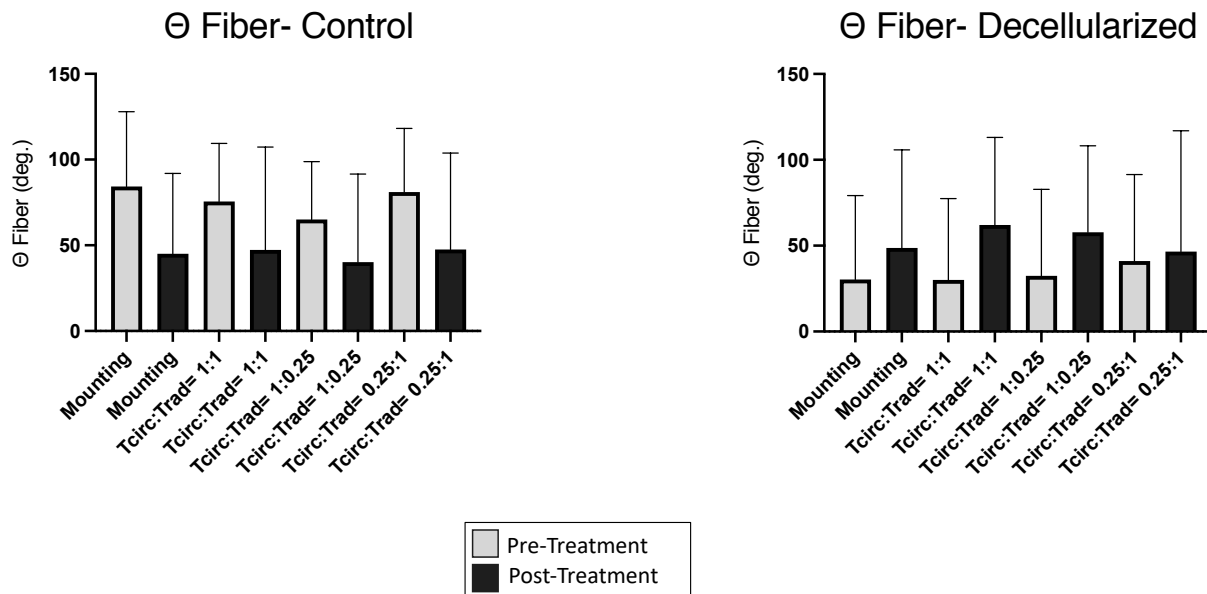
Figure 5-17. θ_{Fiber} values for each loading protocol before treatment and after treatment.

Statistical significance is indicated with $* = p < 0.05$, and $** = p < 0.005$.

Post-treatment θ_{Fiber} values for the control group were also compared to post-treatment θ_{Fiber} values for the decellularized group with a paired t test. No statistically significant results were found from the control group to the decellularized group for any of the loading ratios.
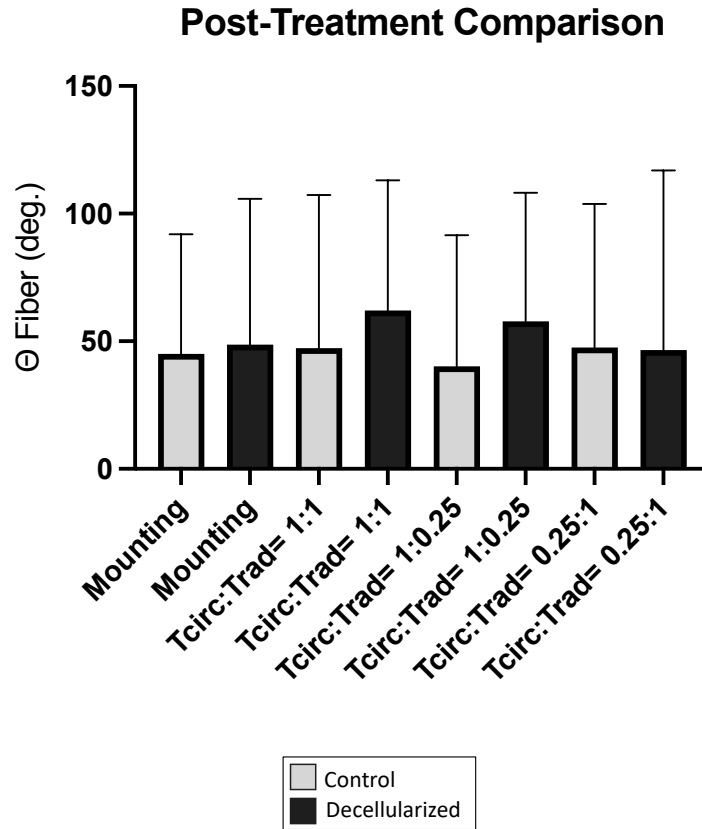
Figure 5-18. θ_{Fiber} post-treatment values from the control group are compared to the post-treatment values from the decellularized group with a paired t test. Statistical significance is indicated with $* = p < 0.05$, and $** = p < 0.005$.

The θ_{Fiber} values were averaged and the percent change calculated between the pre-treatment and post-treatment values (Tables 5-14 and 5-15).

Table 5-15. θ_{Fiber} values and % change from pre-treatment to post-treatment for the control group.

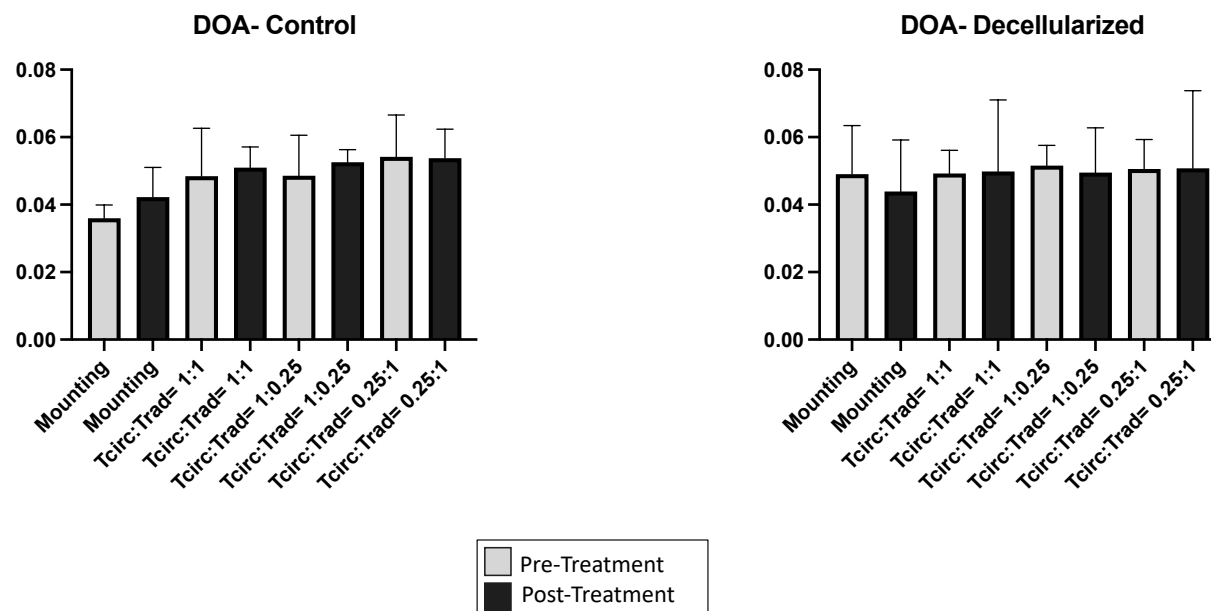| Control								
	Tissue 1	Tissue 2	Tissue 3	Tissue 4	Tissue 5	AVG	SEM	% Change
Mounting, Pre-Treatment	7.72690043	102.448364	94.120553	117.298228	100.029436	84.32469623	19.526317	8%
Mounting, Post-Treatment	111.428554	12.9706619	-4.1096435	31.7353753	73.1531006	45.0356097	20.9987254	
1:1, Pre-Treatment	15.9797076	87.2551034	97.2726243	81.4604726	95.7071125	75.5350041	15.1636792	77%
1:1, Post-Treatment	114.328553	2.58821309	10.7497533	-2.4510351	111.192331	47.28156302	26.8191024	
0.25:1, Pre-Treatment	15.3345513	95.8750082	91.2395383	98.0442103	104.832388	81.06513926	16.5778946	52%
0.25:1, Post-Treatment	119.790476	27.3200318	-22.837197	26.2819908	87.4127945	47.59361924	25.1233524	
1:0.25, Pre-Treatment	7.15535757	74.2914284	93.4078004	81.7910858	68.9453327	65.11820096	15.0611633	52%
1:0.25, Post-Treatment	74.6995816	-15.070983	29.0610432	2.86264467	109.358017	40.18206075	22.9631082	

Table 5-16. θ Fiber values and % Change from pre-treatment to post-treatment for the decellularized group.

Decellularized								
	Tissue 1	Tissue 2	Tissue 3	Tissue 4	Tissue 5	AVG	SEM	% Change
Mounting, Pre-Treatment	36.8254825	61.8716611	-17.666057	-20.173485	90.7955841	30.33063719	21.8487734	17%
Mounting, Post-Treatment	-37.625086	62.4201779	105.574309	88.3974258	24.9252988	48.7384251	25.5108462	
1:1, Pre-Treatment	-8.2422616	68.1232655	-18.334127	18.6238411	89.964606	30.02706473	21.1863271	8%
1:1, Post-Treatment	-20.09817	109.392589	94.3165506	77.1588753	49.4266256	62.03929402	22.823672	
0.25:1, Pre-Treatment	-8.5290893	109.122431	6.76790905	19.0719978	78.6806976	41.0227892	22.5458444	40%
0.25:1, Post-Treatment	-27.955541	98.9243973	85.6045287	108.179066	-32.134298	46.52363065	31.47123	
1:0.25, Pre-Treatment	20.6540158	58.091728	-32.377548	13.7470809	101.943133	32.41168196	22.5591909	0%
1:0.25, Post-Treatment	32.8507327	79.5738241	91.1058112	104.018142	-18.638661	57.78196995	22.5663757	

Pre-treatment DOA values were compared to post-treatment DOA values for each loading protocol for both the control and decellularized groups using a paired t test. No statistically significant results were found from pre-treatment to post-treatment for any of the loading ratios, in either the control group or the decellularized group (Figure 5-19).

Figure 5-19. DOA values for each loading protocol before treatment and after treatment.

Statistical significance is indicated with $* = p < 0.05$, and $** = p < 0.005$.

Post-treatment DOA values for the control group were also compared to post-treatment DOA values for the decellularized group with a paired t test. No statistically significant results were found from the control group to the decellularized group for any of the loading ratios.
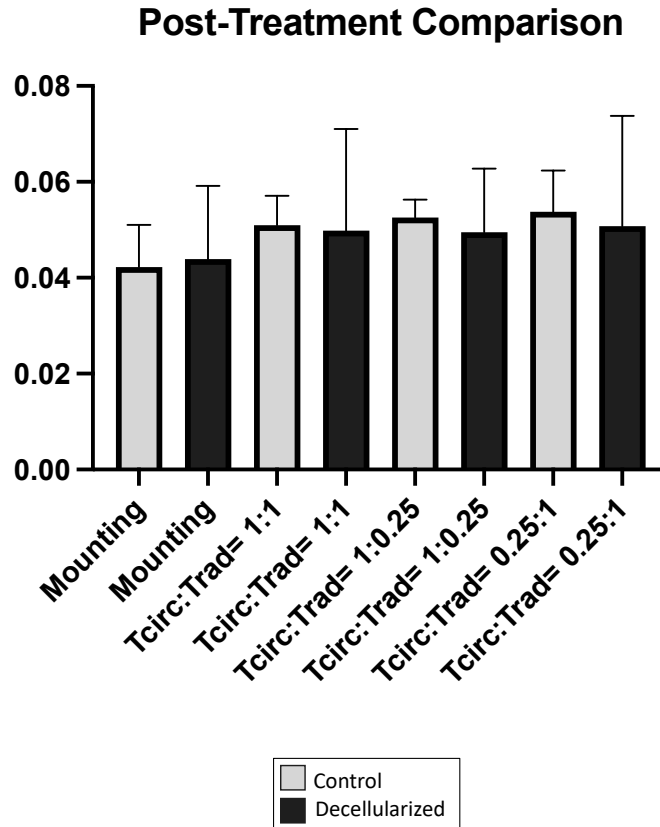
Figure 5-20. DOA post-treatment values from the control group are compared to the post-treatment values from the decellularized group with a paired *t* test. Statistical significance is indicated with ∗ = p < 0.05, and ∗∗ = p < 0.005.

The DOA values were averaged and the percent change calculated between the pre-treatment and post-treatment values (Tables 5-16 and 5-17).

Table 5-17. DOA values and % change from pre-treatment to post-treatment for the control group.

Control								
	Tissue 1	Tissue 2	Tissue 3	Tissue 4	Tissue 5	AVG	SEM	% Change
Mounting, Pre-Treatment	0.04286324	0.03395346	0.03535408	0.03398238	0.03365006	**0.03596064**	**0.00175064**	125%
Mounting, Post-Treatment	0.05399599	0.0487546	0.03687108	0.03285801	0.03852988	**0.04220191**	**0.00394492**	
1:1, Pre-Treatment	0.06132054	0.06062569	0.0390142	0.05244398	0.02871334	**0.04842355**	**0.00635774**	-57%
1:1, Post-Treatment	0.06143713	0.0508745	0.0456644	0.04855617	0.04819562	**0.05094556**	**0.00274991**	
0.25:1, Pre-Treatment	0.07029164	0.06361584	0.04441236	0.05117472	0.04127906	**0.05415472**	**0.00556547**	-31%
0.25:1, Post-Treatment	0.0611084	0.06361766	0.04286106	0.05257408	0.04858284	**0.05374881**	**0.00386114**	
1:0.25, Pre-Treatment	0.05310294	0.06373774	0.04921365	0.04613976	0.03066327	**0.04857147**	**0.00537391**	-69%
1:0.25, Post-Treatment	0.05481477	0.05478718	0.05393153	0.04601705	0.05333598	**0.0525773**	**0.00166337**	

Table 5-18. DOA values and % change from pre-treatment to post-treatment for the decellularized group.

Decellularized								
	Tissue 1	Tissue 2	Tissue 3	Tissue 4	Tissue 5	AVG	SEM	% Change
Mounting, Pre-Treatment	0.06464886	0.04363585	0.06413646	0.03784592	0.03485073	0.04902356	0.0064319	6%
Mounting, Post-Treatment	0.06550218	0.05377333	0.03242236	0.02974766	0.03821248	0.0439316	0.00681361	
1:1, Pre-Treatment	0.05729272	0.03969912	0.04600949	0.05402215	0.04922376	0.04924945	0.00307555	208%
1:1, Post-Treatment	0.05027225	0.06279526	0.030822	0.02772111	0.0776501	0.04985215	0.00946667	
0.25:1, Pre-Treatment	0.0598802	0.04484218	0.0389325	0.05196502	0.05736431	0.05059684	0.00389246	164%
0.25:1, Post-Treatment	0.05935833	0.0669326	0.02379512	0.02894497	0.07472763	0.05075173	0.01027845	
1:0.25, Pre-Treatment	0.06026112	0.04410218	0.05270133	0.05260829	0.04813489	0.05156156	0.00269686	120%
1:0.25, Post-Treatment	0.0576494	0.06005227	0.03484131	0.03517921	0.05971897	0.04948823	0.00592517	

A one-way MANOVA was used to determine if there are any differences between decellularized and control groups for two dependent variables (θ_{Fiber} and DOA). In this study, the independent variable is treatment, which has two groups of decellularized and control. This one-way MANOVA test was repeated for each loading ratio (T_{circ}:T_{rad} = mounting, 1:1, 1:0.25, 0.25:1). There were no statistically significant results for any of the loading ratios, so no post-hoc analysis was attempted (Table 5-19).

Table 5-19. *P*-value results from one-way MANOVA repeated for each loading protocol.

Tcirc:Trad	P Value	Significance
Mounting	0.96	n.s.
1:1	0.92	n.s.
1:0.25	0.85	n.s.
0.25:1	0.96	n.s.

A colormap was generated to demonstrate the θ_{Fiber} and DOA in different regions of the tissue under the four loading ratios for both pre-treatment and post-treatment (Figure 5-21). White lines indicate the average θ_{Fiber} angle for the collagen fibers in the area. The colors represent the DOA, with cooler colors corresponding to higher DOA and less fiber alignment, and warmer colors corresponding to lower DOA and higher fiber alignment.
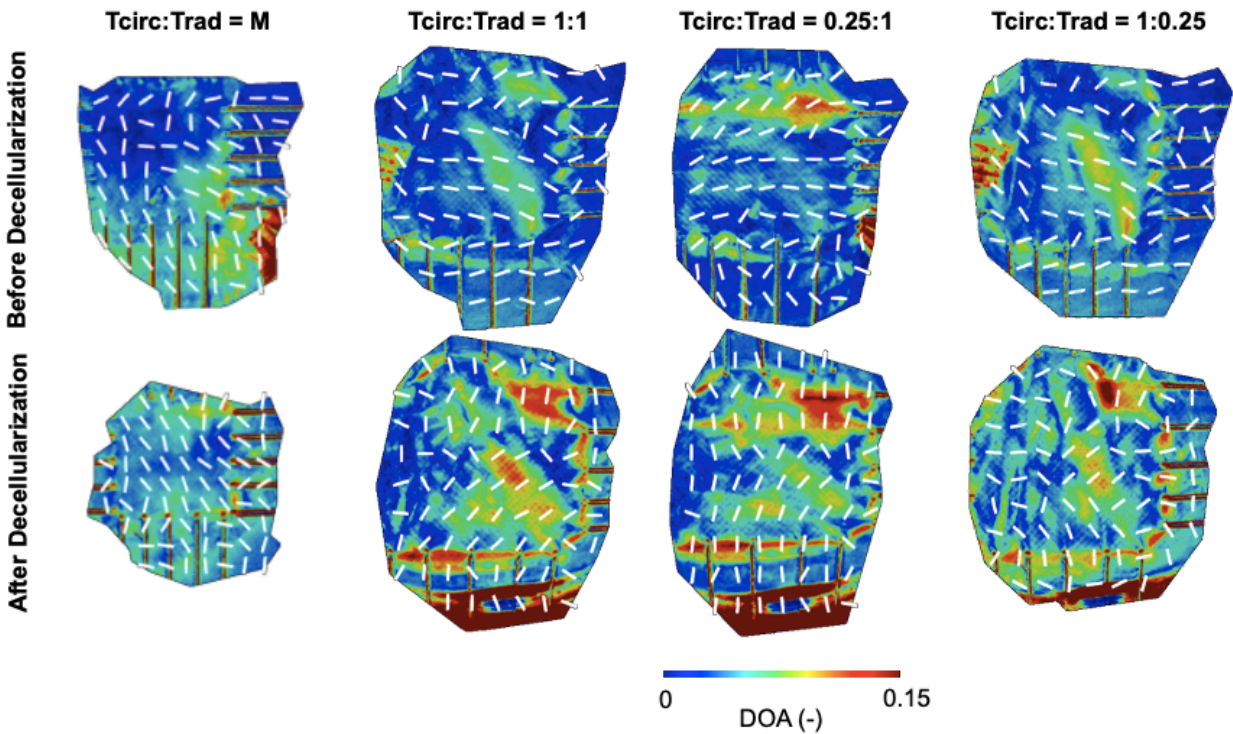
Figure 5-21. Colormap of leaflet before treatment and after treatment under four different loading ratios. White lines represent the average θ_{Fiber} angle of collagen fibers in the area. Colors represent the DOA.

5.4 Discussion

The lack of statistically significant results from pre-treatment to post-treatment for the high tension and low-tension moduli in both the circumferential and radial directions is strong evidence suggesting that the chosen decellularization procedure maintains the mechanical properties of the native valve. Additionally, the control post-treatment to decellularized post-treatment comparison for the high tension and low-tension moduli in both circumferential and radial directions showed no statistically significant results, suggesting that the decellularization reagents do not cause further damage to the microstructure when compared to the DI water and PBS.

There was a general trend of increased peak stretch post-treatment compared to pre-treatment in both the circumferential and radial directions, however since this observation was made for both the control and the decellularized groups, this observation is most likely due to the tissues soaking in solution rather than the decellularization treatment itself.

A one-way MANOVA was implemented to investigate the effects of the treatment (decellularized vs. control) on several parameters including circumferential high-tension modulus, radial high-tension modulus, circumferential low tension modulus, radial low-tension modulus, circumferential peak stretch, and radial peak stretch. This MANOVA was repeated for each loading variable, and no statistically significant results were found for any of the loading conditions. These results suggest the decellularization treatment does not significantly affect any of the measured parameters when compared to the control treatment.

No statistically significant results were seen from pre-treatment to post-treatment for either the control group or the decellularized group, both for θ_{Fiber} and DOA values. Additionally, control post-treatment to decellularized post-treatment tests showed no statistically significant

results for either θ_{Fiber} or DOA. A one-way MANOVA was applied to this data as well, with the independent variable of treatment (decellularized vs. control) and dependent variables θ_{Fiber} and DOA. This MANOVA was repeated for each loading ratio, and there were no statistically significant results observed. From this data, we can conclude that the decellularization treatment does not impact the collagen architecture of the leaflet.

Chapter 6 Conclusions

6.1 Conclusions and Future Work

The optimized decellularization procedure chosen in this thesis (24 h detergents, 12 h enzymes) demonstrated lack of visible nuclei for all three leaflets with a sample size of 3 for each leaflet. Overall, the results from the biaxial testing resoundingly suggested that the chosen decellularization procedure effectively preserves the tissue mechanical properties of the native leaflet. However, due to time constraints, only a sample size of 5 was achieved. More testing should be done to confirm the results reported in this study.

While this study focused solely on leaflet tissue, future studies should be done to attempt decellularization of the whole valve, as this may be what is implanted into patients in the future. Once whole-valve decellularization is accomplished, the graft can be recellularized either in situ or in vitro with cells such as valvular interstitial cells, valvular endothelial cells, mesenchymal stem cells, and others. In addition to recellularization studies, the biaxial/pSFDI analysis – decellularization – biaxial/pSFDI analysis pipeline may be implemented with other tissues undergoing a tissue engineering study.

While more studies undoubtedly need to be done to confirm the findings in this thesis, these results provide an exciting first step toward a issue-engineered heart valve that is hemocompatible, immunologically compatible, and readily remodeled to grow and adapt with the body.

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