Published in final edited form as:

Cell Tissue Bank. 2016 September ; 17(3): 531–539. doi:10.1007/s10561-016-9560-y.

Comparison and Evaluation of Biomechanical, Electrical, and Biological Methods for Assessment of Damage to Tissue Collagen

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Abstract

In regard to evaluating tissue banking methods used to preserve or otherwise treat (process) soft allograft tissue, current tests may not be sufficiently sensitive to detect potential damage inflicted before, during, and after processing. Using controlled parameters, we aim to examine the sensitivity of specific biomechanical, electrical, and biological tests in detecting mild damage to collagen. Fresh porcine pulmonary heart valves were treated with an enzyme, collagenase, and incubated using various times. Controls received no incubation. All valves were cryopreserved and stored at -135°C until being rewarmed for evaluation using biomechanical, permeability, and cell viability tests. Statistically significant time dependent changes in leaflet ultimate stress, ($p=0.006$), permeability ($p=0.01$), and viability ($p=0.02$, 4 different days of culture) were found between heart valves subjected to 0–15 minutes of collagenase treatment (ANOVA). However, no statistical significance was found between the tensile modulus of treated and untreated valves ($p=0.07$). Furthermore, the trends of decreasing and increasing ultimate stress and viability, respectively, were somewhat inconsistent across treatment times. These results suggest that permeability tests may offer a sensitive, quantitative assay to complement traditional biomechanical and viability tests in evaluating processing methods used for soft tissue allografts, or when making changes to current validated methods. Multiple test evaluation may also offer insight into the mechanism of potential tissue damage such as, as is the case here, reduced collagen content and increased tissue porosity.

Keywords

Soft Tissue; Heart Valve; Cryopreservation; Cell Viability; Tensile Modulus; Matrix Permeability

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CONFLICT OF INTEREST

KGMB is an owner and employee of Tissue Testing Technologies, ZC, EDG, and LHC are employees of Tissue Testing Technologies. None of the other authors of this paper have any potential conflicts of interest that might be construed as affecting the conduct or reporting of the work presented.

INTRODUCTION

Each year, more than 100,000 heart valve replacement procedures are performed in the United States (Mozaffarian et al. 2015). While mechanical prostheses remain popular, xenogeneic and allogeneic valves are important for certain patient groups such as children with congenital heart disease and geriatric patients with poor adherence to the medications necessary for mechanical valves (Brockbank et al. 2015). Processing methods for these bioprosthetic valves should minimize damage that may cause replacement failure. Sensitive tests are needed to detect this damage. However, the tissue banking profession has a marked tendency to employ analytical tests for soft tissues during development of processing methods without demonstration that the tests can actually detect change in the target tissues induced by potential damage mechanisms.

Heart valve leaflets are composed of three defined layers with distinct ECM structures that are essential for biomechanical function (Hinton and Yutzey 2011; Sacks et al. 2009). The atrialis/ventricularis layers face the inflow and are mainly composed of elastin that allows for flexible movement of the leaflets during the cardiac cycle. The spongiosa is the middle layer of loose collagen fibers surrounded by proteoglycans (PGs) and glycosaminoglycans (GAGs). Last, the fibrosa faces the outflow and is mainly composed of fibrillar collagen that provides strength and stiffness that is necessary to seal the valve and prevent backflow during diastole (Sacks et al. 2009). Damage to the fibrosa and its collagen structure may lead to valve failure. Collagen comprises one-third of the total protein and is the most prevalent component of the extracellular matrix (ECM) in humans (Shoulders and Raines 2009). Collagen is responsible for the proper structure and function of many tissues.

We employed porcine heart valve leaflets in this study. Our objective was to compare a selection of available heart valve leaflet tissue test methods to determine whether or not relatively mild collagen damage could be detected using a collagenase-induced damage model. While we focus on heart valve tissue in this study, the principles can be applied to the matrix integrity of other soft tissues. The damage model was based upon that employed by Votteler *et al.* (Votteler et al. 2012) in which Raman spectroscopy was used to detect ECM damage in porcine heart valve leaflets caused by as little as 15 minutes of exposure to collagenase. Increasing collagen degradation induced by longer collagenase exposure times translated into decreasing Raman signal intensities across the whole spectrum, as these modifications result from changes in the composition and configuration of collagen molecules and hydrogen bonding. This model simulates damage that could occur due to release of endogenous enzymes during procurement and processing of heart valves and other soft tissues such as veins, arteries, fascia lata, amniotic membrane, pericardium, skin, ligaments and tendons. In this study, we assessed the effect of this damage model on leaflet mechanics and compared the results with cell viability and a novel tissue permeability assay based upon electrical conductivity.

MATERIALS AND METHODS

Porcine pulmonary heart valve preparation

A total of 34 pig hearts were procured from a local slaughter house following the current standards of the American Association of Tissue Banks for human cardiac tissue recovery, ischemia and preservation (AATB 2012). The hearts were rinsed and transported on ice in lactated Ringer's solution. The pulmonary heart valves were then dissected under aseptic conditions, treated with antibiotics by means of Dulbecco's modified Eagle medium (DMEM; Mediatech, Herndon, Va., USA) containing 4.5 g/l glucose and 1% penicillin-streptomycin (Sigma, St. Louis, Mo., USA) at 4°C overnight. The valve leaflets with a section of artery connected by a muscle band were dissected from each valve. One leaflet segment from each valve served as an untreated control while the other two were randomized to treatment groups. Treatment consisted of leaflet exposure to collagenase for 2.5, 5, 10, and 15 minutes at 37°C. The longest duration corresponded to that under which Votteler *et al.* demonstrated collagen damage detectable by RAMAN spectroscopy. The collagenase was 1000 U/mL Liberase according to Mandl, which is equal to 1 PZ U/ml according to Wünsch (Mandl et al. 1953; Wuensch and Heidrich 1963). The incubation was stopped with an ice cold solution and the tissues were rinsed twice. All tissue samples, both control and treated were then cryopreserved using 10% DMSO in DMEM and slow rate cryopreservation, as previously described in the literature (Brockbank et al. 2011a), in Nunc™ cryogenic storage tubes. The samples were stored at -135°C and rewarmed for evaluation in small manageable groups. Samples were thawed by immersion in a 37°C water bath until all ice had visibly disappeared. The cryoprotectant formulation was then removed and the tissues washed in ice cold DMEM culture medium with 0.5 M mannitol for 15 min, 0.25 M mannitol for 15 min, and then mannitol-free DMEM for 15 min. Finally, the DMEM was exchanged for fresh DMEM and the tissues were prepared for testing (Figure 1).

Biomechanical testing

Biomechanical test was performed on a soft tissue mechanical testing system (Bose ELF 3200; Bose Corporation, Eden Prairie, MN) to evaluate tensile properties of 4.0 mm wide radial strips based upon published testing and calculation methods (Lee and Boughner 1981; Lee et al. 1984; Vesely et al. 1990) as previously described (Brockbank et al. 2011c). Samples were tested at room temperature and kept hydrated during testing using phosphate buffered saline. An initial tare load of 0.02N was applied to the tissue for preconditioning and the sample was loaded at a rate of 10 mm/minute until failure. Failure was considered to be a 20% reduction in force. Data were recorded as force versus displacement. The initial gauge length of the specimen was measured after preconditioning and used for calculating strain. The initial gauge length was approximately 5.0 mm for the samples tested. The initial cross-sectional area measured with a custom-designed current sensing micrometer during dissection was used for stress calculations. Young's modulus (E) was calculated as the slope of the linear elastic portion of the stress-strain curve. Ultimate stress, the maximum stress recorded before failure, was also determined for each sample.

Permeability assay

Permeability assessment of 5 mm diameter punch biopsies was performed in isotonic PBS solution using an electrical conductivity assay as previously described (Brockbank et al. 2011b). The electrical conductivity was measured based on the principle of a four wire resistance test using a Keithley Sourcemeter (Model 2400, Keithley Instruments, Inc., Cleveland, OH) and a custom designed conductivity chamber reported previously (Brockbank et al. 2011b; Gu et al. 2002). Briefly, the conductivity apparatus consists of two stainless steel current electrodes coaxial to two Teflon-coated Ag/AgCl voltage electrodes placed on the top and bottom of a cylindrical nonconductive Plexiglass chamber (5mm diameter). The specimen was placed inside the chamber for measurement. The resistance (R) values across the specimens were measured at a low, constant DC current density of 0.015 mA/cm². The height of the specimen was measured with an electrical current sensing micrometer. The electrical conductivity (χ) values of the specimens were calculated by:

$\chi = \frac{h}{R \cdot A}$, where h and A are the height and cross-sectional area of the specimens, respectively. The precision for the resistance measurements was 0.5 Ω while the height measured with an accuracy of ± 1.0 μm . All electrical conductivity measurements were performed at room temperature (22°C).

Viability assay

Viability assessment employed the resazurin assay to measure metabolic activity (Brockbank et al. 2011a; O'Brien et al. 2000). The resazurin assay incorporates a water-soluble fluorometric viability oxidation-reduction indicator which detects metabolic activity by both fluorescing and changing color in response to chemical reduction of the growth medium (O'Brien et al. 2000). Tissue samples were incubated for 3 h with resazurin working solution at 37°C, and aliquots of medium were then placed in microtiter plate wells and read on a microtiter plate spectrofluorometer at a wavelength of 590 nm. The results were normalized as relative fluorescent units (RFU)/mg dry weight after subtraction of background fluorescence.

Scanning Electron Microscopy

To prepare samples for SEM, valves were first punched into 5 mm diameter buttons and fixed with 4% paraformaldehyde for 30 minutes. Samples were then cryomicrotomed approximately 100 microns into the tissue to expose the collagen structure and dehydrated through graded ethanols and dried using hexamethyldisilazane. Samples were sputter coated with a palladium gold mix (Pd/Au) and viewed under a scanning electron microscope at 15 kV and 2000X.

Statistics

The samples for biomechanics and permeability testing were blinded so that the test operator could not discern between samples. The data were statistically evaluated by analysis of variance (ANOVA) and Tukey's post hoc tests using SAS software, version 9.2 for XP-Pro (Cary, NC, USA). Statistical differences were reported at P-values < 0.05.

RESULTS

Biomechanical testing

The results of the biomechanical tests showed a general trend of decreasing ultimate tensile stress with increasing collagenase incubation time (Figure 2). Average ultimate tensile stresses with standard deviations for 0, 2.5, 5, 10, and 15 minutes of incubation time were 0.28 ± 0.03 , 0.18 ± 0.04 , 0.19 ± 0.06 , 0.13 ± 0.03 , and 0.16 ± 0.04 MPa, respectively. And while the difference was statistically significant by ANOVA ($p=0.006$), this trend was not consistent across all time points. The decrease in ultimate stress after 10 minutes of collagenase incubation was statistically significant when compared to untreated controls (post-hoc Tukey's, $p=0.004$). The impact of collagenase on tensile modulus, shown in Figure 3, was not significant (ANOVA, $p=0.07$) and no trend was apparent. Average tensile moduli with standard deviations for 0, 2.5, 5, 10, and 15 minutes incubation time were 0.45 ± 0.15 , 0.32 ± 0.08 , 0.48 ± 0.19 , 0.28 ± 0.09 , and 0.38 ± 0.14 MPa, respectively. For both ultimate tensile stresses and tensile moduli, $N=6$ for all groups except for untreated valves (0 min) for which $N=9$, and for ultimate stress of valves treated for 5 minutes for which $N=5$.

Permeability assay

Permeability assessment using electrical conductivity, shown in Figure 4, demonstrated consistently increasing permeability with collagenase incubation time. The increase appeared to be linear (Pearson's $R=0.95$). Average conductivities with standard deviations for 0, 2.5, 5, 10, and 15 minutes of incubation time were 6.41 ± 1.47 , 7.17 ± 1.04 , 7.66 ± 1.45 , 8.05 ± 1.90 , and 9.19 ± 1.24 mS/cm, respectively. The difference between groups was statistically significant by ANOVA ($p=0.01$). The increase in permeability after 15 minutes of collagenase incubation was statistically significant when compared to the permeability of the untreated controls (post-hoc Tukey's, $p=0.005$). For all groups, $N=6$ except for untreated valves (0 min) for which $N=12$.

Viability assay

There was also a marked trend for viability to increase with collagenase incubation time, as shown in Figure 5. At day 0, average viability with standard deviations for 0, 2.5, 5, 10, and 15 minutes incubation time were 2549 ± 524 , 3649 ± 917 , 2899 ± 738 , 4247 ± 1200 , and 4678 ± 1559 RFU/mg of tissue, respectively. The difference in groups was statistically significant by ANOVA for all days ($p<0.0001$, $p=0.01$, $p=0.009$, and $p=0.02$ for days 0, 1, 2, and 3, respectively). By post-hoc Tukey's, increases in viability following collagenase incubation were significant when compared to untreated controls for day 0: 10 min ($p=0.002$), day 0: 15 min ($p<0.0001$), day 1: 10 min ($p=0.02$), and day 2: 15 min ($p=0.03$). There were no significant decreases in viability observed during incubation under tissue culture conditions up to 3 days ($p=0.73$). This observation suggests there was little or no apoptosis induced by the collagenase treatments. $N=8$ for all groups and days except for the untreated tissue (0 min), for which $N=16$ for all days.

Scanning electron microscopy

Images obtained from SEM are shown in Figure 6. Only two groups were imaged: controls and heart valves leaflets treated with the maximum 15 minutes of collagenase incubation. Collagen fibers appear to be somewhat disconnected and less compact in the treated valves when compared to the control. However, this difference was difficult to detect and, thus, imaging was not performed on samples from the intermediate treatment time groups. Also, no quantitative image analysis was performed.

DISCUSSION

The tissue banking industry often employs tests in developing processing methods without verifying the sensitivity of such tests to potential damage. Heart valves are a commonly transplanted tissue that may suffer loss of function and/or failure due to collagen damage. In this study, we evaluated the sensitivity of some common tests as well as a novel conductivity test with a collagen damage model on porcine pulmonary valve leaflets. We chose relatively short incubation times with collagenase to determine which tests were sensitive to minimal damage.

Our results demonstrated a significant difference in ultimate stress but no difference in tensile modulus and suggest that the biomechanical test methods alone, as employed, may not be sensitive enough to reveal the full impact of collagenase treatment over such a short time frame. While great care was taken in sample preparation, consistency in measurements for cross-sectional area is tedious, and errors can have a large impact on the final calculated stresses and moduli. It is also possible that collagenase effects are being masked by cryopreservation-induced ECM changes since cryopreservation has been demonstrated to induce increases in biomechanical properties such as burst pressure in tissue engineered blood vessels (Dahl et al. 2006; Elder et al. 2005).

Our results should not be interpreted as indicating that biomechanical tests have no value for assessment of tissue damage. Longer collagenase treatments or higher collagenase concentrations or alternative enzyme formulations, such as trypsin (Perie et al. 2006), or alternative damage models such as heat or chemical treatment would eventually demonstrate a full range of biomechanical changes. However, as indicated above, biomechanical assays may not always be sensitive enough to reveal an impact of “mild” collagenase treatments, as we employed here, or other types of damage that may be encountered in tissue processing for banking and clinical use. Therefore, alternative complementary assays should be performed, the nature of which will depend on the type of tissue and tissue attributes about which there is potential concern.

These observations led to evaluation of matrix permeability. Electrical conductivity is a material property of biological tissues. Its value is related to the diffusivity of small ions in the tissue, which depend upon tissue composition and structure (Frank et al. 1990; Maroudas 1968). Using an electrical conductivity method, the effect of matrix composition on solute permeability has previously been studied in hydrogels and cartilaginous tissues (Gu et al. 2004; Jackson and Gu 2009; Kuo et al. 2011). These studies show that the electrical conductivity is positively correlated with tissue porosity (i.e., water volume fraction) in

cartilaginous tissues (e.g., articular cartilage, intervertebral disc, and temporomandibular joint disc). The increased conductivity after collagenase treatment was likely due to increased porosity and water content. As collagen fibers are degraded, the tensile strength of the leaflets is reduced and is less able to counterbalance the Donnan osmotic swelling pressure caused by the fixed charge on the PGs and GAGs of the spongiosa. Increased water content increases small ion diffusivity, which is detected by our conductivity method.

Viability has historically been used for evaluation of cryopreserved human allograft heart valves destined for clinical use. Many means of assessing cell viability have been described for heart valve tissues including amino acid uptake, protein synthesis, contractility, dye uptake, ribonucleic acid synthesis, 2-deoxyglucose phosphorylation and the resazurin metabolism assay (Brockbank et al. 1992; Brockbank and Dawson 1993; Brockbank et al. 2011a; Hu et al. 1990; Messier et al. 1994; Mochtar et al. 1984; Watts et al. 1976). We employed the metabolism-based resazurin assay (O'Brien et al. 2000) because it has an advantage over other more commonly employed assays due to being non-cytotoxic and non-destructive, so the same piece of tissue can be assayed several times, as we have done in this study, provided that it is maintained under physiological tissue culture conditions. However, the resazurin assay does not discriminate between tissue and cell types in samples. At present the hypothetical link between the extended performance of cryopreserved heart valves and viability is not the persistence of donor cells *in vivo*, but rather that viability correlates with gentle treatment of the valves *in vitro* resulting in better *in vivo* function because better matrix preservation has occurred, and the donor cells die via apoptosis rather than necrosis, which incites a gross inflammatory response (Wolfenbarger et al. 2004). We were not surprised by the absence of a decrease in cell viability after 2.5 – 15 minute collagenase treatments. Collagenase treatments are commonly used for isolation of cells from tissues and removal of adherent cells from substrates during routine tissue culture. We were, however, surprised by the observed trend towards an increase in metabolic activity with duration of collagenase treatment compared with untreated controls. A likely hypothesis to explain the observed increase in metabolic activity could be better access to nutrients. Collagen damage may result in higher water content and improved permeability and diffusion of nutrients or viability assay reagent. There were no decreases in viability observed during incubation under tissue culture conditions suggesting there was little or no apoptosis induced by collagenase treatments.

The results of the SEM agree with and support increased tissue porosity when exposed to increased incubation time with collagenase. The collagen fibers of the treated valves appear loose and less compact when compared to untreated controls. However, this qualitative difference is difficult to detect, and SEM should not be employed as a stand alone test in preventing damage during development of tissue processing methods.

The highly organized ECM of the mature valve is composed of water, collagen, elastin, and proteoglycan (Hinton and Yutzey 2011). From a biomechanical point of view, similarly to other soft tissues, vascular tissue is a biphasic material, which is composed of a solid (matrix proteins) and a fluid (interstitial fluid) phase. The mechanical response of the tissue to external loading is therefore determined by the interaction between its solid and fluid domains, which translates to the interstitial fluid flow through the porous deformable solid

phase (Swartz and Fleury 2007). During the normal cardiac cycle, the aortic valve leaflet routinely withstands large deformations such as changes in area as high as 50% (Schoen and Levy 1999). This indicates a significant role of interstitial fluid flow in mechanical function of the leaflet (Wang et al. 2011) as in the case of other soft tissues such as cartilage and intervertebral discs (Garcia et al. 1996). Therefore, the permeability characteristic is an important mechanical property of heart valves that is often overlooked in contrast to more commonly studied tensile or compressive parameters. Our results demonstrate that leaflet tissue permeability was impacted in a time dependent manner by collagenase treatment. Permeability testing promises to provide a sensitive quantitative test that can be employed for validation of process changes for soft tissues.

CONCLUSIONS

While ultimate stress was decreased by collagenase treatment, it was not changed in a consistent manner and no change was found in tensile modulus. This observation supports the conclusion that biomechanical tests alone may not be sufficient for detection of mild to moderate changes induced by collagen degradation. In contrast, tissue permeability was impacted in a time dependent manner by collagenase treatment. Viability assessment using a metabolic assay also increased. This increase was probably a consequence of the increased tissue permeability induced by the collagenase treatments. Combined biomechanical, permeability, and viability testing, in the case of tissues with live cells, promises to provide quantitative tests that can be employed in evaluating processing methods used for soft tissue allografts, or when validating changes to current methods used by tissue banks.

Acknowledgments

FUNDING

This research was supported by a grant from the Scientific and Technical Affairs Committee of the American Association of Tissue Banks (to KGMB and AL-J) and National Institutes of Health grants DE021134, DE018741, and AR055775 to HY, and a National Institutes of Health F31 predoctoral fellowship DE023486 to GJW.

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Fig. 1. Sample preparation and testing of heart valve leaflets. (A) From each pulmonary heart valve, one leaflet was used for control and the other two were randomized to treatment groups. (B) Leaflets from the control group and each treatment group were dissected as shown and subjected to biomechanical, permeability, and tissue culture viability tests

Fig. 2.

Mean ultimate stresses (before failure) and standard error bars for heart valves of different collagenase treatment times. Failure was determined as a 20% reduction in force. N=6 for all treated groups except for 5 min, for which N=5. N=9 for controls (0 min). ANOVA, $p = 0.006$. *Post-hoc Tukey's comparison with control: $p = 0.004$

Fig. 3. Tensile Young's modulus and standard error bars for heart valves of different collagenase treatment times. Moduli were calculated from the slope of the linear portion of the stress-strain curves. N=6 for all treated groups. N=9 for controls (0 min). ANOVA, $p=0.07$

Fig. 4. Conductivity and standard error bars of heart valves of different collagenase treatment times. N=6 for all treated groups. N=12 for controls (0 min). ANOVA, $p=0.01$. *Post-hoc Tukey's comparison with control: $p=0.005$

Fig. 5. Metabolism assay for heart valves, with standard error bars, of different collagenase treatment times cultured for 0,1,2, and 3 days. N=8 for all treated groups. N=16 for controls (0 min). ANOVA, p 0.02. *Post-hoc Tukey's comparison with control: p 0.03

Fig. 6. SEM images of heart valves. Collagen fibers of heart valves treated for 15 minutes incubation with collagenase appear loose in some areas (black arrow) when compared to fibers in the controls