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Mechanical characterisation of a customised decellularized scaffold for vascular tissue engineering

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Highlights

- Decellularized arterial tissue as a scaffold for vascular tissue engineering.
- Customisation of decellularized tissue by creating capability for bulk cell seeding the medial layer.
- Medial cavity creation and controlled collagen digestion does not sacrifice mechanical properties of the tissue.
- Creation of a novel scaffold for complete cell repopulation with reduced seeding/culture times.

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MECHANICAL CHARACTERISATION OF A CUSTOMISED DECELLULARIZED SCAFFOLD FOR VASCULAR TISSUE ENGINEERING

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Abstract

Several challenges persist when attempting to utilise decellularized tissue as a scaffold for vascular tissue engineering. Namely: poor cell infiltration/migration, excessive culture times associated with repopulating the scaffolds, and the achievement of a quiescent medial layer. In an attempt to create an optimum vascular scaffold we customised the properties of decellularized porcine carotid arteries by: (i) creating cavities within the medial layer to allow direct injection of cells, and (ii) controlling the amount of collagen digestion to increase the porosity. Histological examination of our customised scaffold revealed a highly porous tissue structure containing consistent medial cavities running longitudinally through the porous scaffold wall. Mechanical testing of the customised scaffold showed that our minimal localized disruption to the ECM does not have a detrimental effect on the bulk mechanical response of the tissue. The results demonstrate that an increased stiffness and reduced distensibility occurs after decellularisation when compared to native tissue, however post scaffold customisation we can revert the scaffold tensile properties back to that of native tissue. This most noteworthy result occurs in the elastin dominant phase of the tensile response of the scaffold, indicating no disruption has occurred to the elastin network by our decellularization and customization techniques. Additionally, the bulk seeding potential of the customised scaffold was demonstrated by direct injection of human smooth muscle cells through the medial cavities. The optimum cell dispersion was observed in the highest porosity scaffold, with large cell numbers retained within the medial layer after 24 h static culture. In summary, this study presents a novel customised decellularized vascular scaffold that has the capability of bulk seeding the media, and in tandem to this method the porosity of the scaffold has been increased without compromising the mechanical integrity.

1. Introduction

The prevalence of vascular disease continues to increase worldwide and treatment for which relies heavily on diseased vessel replacement (Roger et al., 2011). Bypass grafting procedures largely utilise the saphenous vein or mammary artery for autologous grafting (Buxton et al., 2009). However, underlying vascular disease or previous vessel harvest often render these vessels unavailable (Eagle et al., 1999, Gray et al., 2008). Subsequently in a large patient cohort this necessitates the need for a suitable alternative to autologous vessel grafting for small diameter applications (<6mm diameter). Substantial progress has been made in the field of tissue engineering to address this clinical need (L'Heureux et al., 2006, Niklason et al., 1999, Zhao et al., 2010), but there still remains some limitations to its progress: obtaining suitable architecture, appropriate mechanical properties, sufficient smooth muscle cell (SMC) density and correct phenotype are still major challenges (Tranquillo, 2002, Villalona et al., 2010, Yazdani et al., 2009).

The arterial wall's unique mechanical properties are obtained by the interaction between the two major extracellular matrix (ECM) proteins, collagen and elastin (Holzapfel et al., 2000, O'Connell et al., 2008, Roach and Burton, 1957). Mimicking their complex network by attaining ECM components in sufficient quantities and levels of maturity within a construct may be a central component of a tissue engineered vessel. Many research groups have successfully matured constructs *in vitro* with high levels of collagen (Hoerstrup et al., 2001, Liu et al., 2007, Niklason et al., 1999). This collagen network provides the overall tensile strength thus creating a strong construct that can withstand implantation and high burst pressures *in vivo*. Elastin fibers organize into concentric sheets of elastic lamellae within the vessel wall and provide the elasticity to the vessel which is essential for compliance. The natural intricate structure of the elastic lamellae and its interaction with collagen and SMCs has resulted in few constructs expressing mature elastin in its native configuration (Dahl et al., 2007, Patel et al., 2006). This is a major limitation in tissue engineering vascular grafts as compliance mismatch *in vivo* is one of the main failure modes for implanted constructs (Kakisis et al., 2005, Wang et al., 2007). This limitation potentially warrants the use of decellularized tissue as the construct of choice for future tissue engineered graft applications.

Decellularized tissue has been used extensively as a scaffold for tissue engineering vascular constructs and may offer the ability to overcome some of the challenges facing current fabrication (Amiel et al., 2006, Schaner et al., 2004, Zhu et al., 2008). These porous three-dimensional structures present the preformed complex architecture of the native vessel and are ideal for cell attachment and growth while maintaining the mechanical integrity of the native tissue (Gilbert et al., 2006). The disruption caused by removing the cellular material from the tissue can be minimized in order to create a stable and robust scaffold. A major drawback, however, is that the matrix that remains after decellularization is extremely dense and can prove difficult to infiltrate with cells. This is particularly the case with SMCs in the medial layer of the construct (Lu et al., 2004, Neff et al., 2010). A poorly infiltrated scaffold resulting in a lack of a fully quiescent contractile SMC medial layer has resulted in less than ideal mechanical properties and diminished performance *in vivo* (Yazdani et al., 2009). The highly dense nature of decellularized vascular tissue has also lead to excessive cell seeding and culture times to mature these constructs *in vitro*. These excessive maturation times are unfeasible if tissue engineering vascular constructs are to have meaningful clinical success.

The aim of this research is to create and mechanically characterise customised decellularized porcine arterial tissue for use as a scaffold for vascular tissue engineering small diameter grafts. The study examines two customisation techniques, the inclusion of medial cavities and small collagen fiber digestion. These techniques are designed to maintain scaffold strength and potentially overcome current tissue engineering problems/deficiencies of unsuitable mechanical properties, incomplete cell seeding and excessive culture times.

2. Material and methods

2.1 Tissue Harvest

Carotid arteries were freshly harvested from 70-90kg pigs in a local abattoir (Lislin Meats Ltd, Mullagh, Co. Cavan, Ireland). Common carotid arteries 5-7mm in diameter were extracted from each pig using sterile instruments and subsequently rinsed in phosphate buffered saline (PBS). All arteries were returned to the laboratory stored in PBS on ice. Samples were prepared by removing excess connective and adventitial tissue and cut into approximately 30mm long segments. All samples were then frozen in PBS for later use (it has been previously shown frozen storage does not significantly affect artery mechanics (Stemper et al., 2007). Samples were divided into four groups for investigation: (i) Native tissue (ii)

Decellularized tissue (iii) Decellularized tissue with medial cavities and (iv) Collagen digested tissue.

2.2 Decellularization

The method used to decellularize the arterial tissue was adapted from a protocol previously described in the literature (Amiel et al., 2006). The protocol consists of an enzymatic digestion and detergent extraction. Firstly, samples were immersed in de-ionized water for 24 h at 4°C. Samples were then incubated in 0.05% Trypsin with 0.02% EDTA (Sigma-Aldrich, Ireland) for 1 h at 37°C. After a short rinse in PBS to remove excess trypsin, the samples were placed in a solution of 2% TritonX-100 and 0.8% ammonium hydroxide (Sigma Aldrich, Ireland) in de-ionized water for 72 h at 4°C. To ensure the elimination of all disrupted cellular material this solution was changed every 24 h. A final wash sequence of 48 h in de-ionized water with a water change after 24 h was carried out to remove all residual chemicals used in the decellularization process. Each step in the above protocol (except trypsin incubation) was carried out under constant rotational agitation to maximize chemical exposure to the tissue.

2.3 Decellularized tissue with micro-needles/medial cavities

Further tissue manipulation was undertaken by the insertion of three small bore micro-needles (Ø300 μ m) into the transverse section of the native tissue. These needles were inserted at approximately 120° circumferentially to each other into the arterial wall and directed longitudinally along the sample within the medial layer to a distance of 12mm from the insertion point (Fig 3 A). The samples were then subjected to the same decellularization protocol as described above.

2.4 Collagen Digested Tissue

To investigate reducing the density of the decellularized tissue further customisation was carried out by removing varying amounts of collagen after the decellularization process. Sodium hydroxide (NaOH) was used to digest the collagen. NaOH has been used previously to digest collagen in preparation of samples for scanning electron microscopy (Crissman, 1987). The above decellularization protocol was followed with one additional step of subjecting the samples to sonication in 0.5M NaOH prior to the final wash sequence. Two groups were subjected to sonication for 15 mins and 90 mins respectively. A third group was

sonicated for 120 mins combined with mechanical agitation in 0.1M NaOH for 24 h at room temperature.

2.5 Mechanical Testing

Uniaxial tensile tests were carried out to determine the mechanical response of the native and decellularized tissues using a Zwick tensile testing machine (Zwick Z005, Roell, Germany).

The following groups ($n=10$ each) were tested:

1. Native tissue.
2. Decellularized tissue.
3. Decellularized tissue with medial cavities.
4. Collagen digested tissue
 - a. 15 mins sonication.
 - b. 15 mins sonication with medial cavities.
 - c. 90 mins sonication.
 - d. 120 mins sonication and 24 h mechanical agitation.

Two ring sections 4mm long were cut from each sample and tensile tested to failure using a 100N load cell. Custom made grips were machined which contained 1mm diameter protruding stainless steel rods which aligned parallel for mounting the ring sections. The tests were undertaken at a constant displacement rate of 2mm/min in a PBS bath at room temperature. All samples were preconditioned to a crosshead displacement of 0.1mm (corresponding to ~10% strain). Tissue displacement was determined by the use of video extensometer tracking of dots applied to the transverse wall of the ring section. These dots (approximately 1mm diameter) were applied with red permanent marker to the wall section roughly 1mm apart. Two dots were applied on each side of the sample wall in this manner and aligned parallel when placed on the bars of the grips of the tensile testing machine (Fig 3B). The video was triggered at the commencement of movement of the crosshead and a custom frame grabbing program recorded images of the test at a rate of 1frame/second with a time stamp embedded in the recorded file name. This allowed for the tracking of the dots on the tissue and their relative displacement calculated by analysing the recorded images. ImageJ software (US National Institutes of Health) was used to determine the centroid of each dot for each individual frame and hence calculate the relative displacement.

True stress (σ) and true strain (ϵ) were calculated using the following formulae:

$$\sigma = \frac{F(t)}{A_0 \frac{L_0}{L(t)}} \quad (1)$$

$$\varepsilon = \ln \left(\frac{L(t)}{L_0} \right) \quad (2)$$

Where $F(t)$ is the measured force at time t , A_0 is the initial cross-sectional area, L_0 is the initial displacement (distance between two vertical dots in the first frame) and $L(t)$ is the displacement at time t . L_0 is determined after the preconditioning cycle by manually moving the crosshead until the dots align vertically and parallel to the central axis of the test machine. The test commenced once the force was zeroed in this position. L_0 was determined in this manner due to the variability within samples in the small strain region of the stress-strain curve. This variability is largely due to the dimension variations (diameters and wall thickness vary slightly between samples). The displacement between the dots is measured by calculating the vertical distance between the centroid of each dot.

The mechanical response of the tested samples was observed by generating the stress-strain curves from the tensile test data. All data was averaged for the applied 3rd order fitted polynomial equation to the test data at strain intervals up to the maximum strain at failure for each group. For each group described above the strain at failure and ultimate tensile strength (UTS) was also recorded. The values for the modulus of the collagen and elastin dominant phases were determined by calculating the slope of the linear regression curve within these regions as described below (see Fig 3D for an example of determining the moduli of the two regions).

2.6 Scaffold Repopulation

Human aortic smooth muscle cells (hSMCs, Lonza, UK) were cultured in standard tissue culture flasks using Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, Ireland) supplemented with 20% foetal bovine serum, 1% L-glutamine and 2% penicillin/streptomycin. Media was changed every 3 days and cells were removed from flasks using trypsin-EDTA solution. Cells were expanded and all seeding experiments were undertaken between passages 6-10.

Decellularization of porcine carotid arteries was carried out as described in section 2.2 and three micro-needles were inserted into the tissue to create medial cavities for cell delivery. 10 mm long scaffolds of decellularized tissue (no collagen digestion), 15 mins sonication in

NaOH and 90 mins sonication in NaOH were prepared for initial investigation of the feasibility of cell seeding within the medial cavities ($n=2$ each). 30 mm long scaffolds of 90 mins sonication in NaOH were also prepared ($n=2$). All processing steps were carried out under sterile conditions in a laminar flow hood to reduce the risk of contamination of the scaffolds. Two hours before cell injection the scaffolds were soaked in sterile cell culture media (DMEM) and incubated at 37° C with 5 % CO₂.

The 10 mm long constructs were injected with hSMCs at a concentration of 500,000 cells/0.05ml media in each needle. The needles were slowly removed from the tissue as the cells were simultaneously injected through the medial cavities. Injected scaffolds were left standing vertically for 30 mins to allow for cells to settle and to begin adhesion to the scaffold. After 30 mins cell culture media was added and the constructs were incubated for 2 h.

The 30 mm long scaffolds were injected with 1×10^6 cells/0.1ml media in each needle in the same manner. After 30 mins the injected scaffolds were incubated for 24 h.

2.7 Histology

5mm ring segments were taken from each tested sample group for histological analysis. Samples were embedded in paraffin wax in an automatic tissue processor (ASP300, Leica, Germany). The needles were removed from samples before processing of the decellularized tissue with medial cavities group. All samples were sectioned longitudinally using a rotary microtome (Leica microtome, Leica, Germany). 7 μ m sections were cut and collected on glass slides and subsequently washed through a graded series of ethanol from 100 to 70% (v/v). Samples of native and decellularized tissue were stained using Hematoxylin and Eosin (H&E), Masson's Trichrome and Picrosirius Red. To further verify cell removal sections were also stained with diamidino-2-phenylindole (DAPI). Sections were dehydrated and cleaned in ascending concentrations of ethanol and xylene before coverslips were mounted (DPX mountant, BDH). Observation under light microscopy and digital image acquisition was carried out with an inverted microscope (Olympus IX 71).

Cell injected scaffolds were embedded in OCT compound (Tissue Tek) and snap frozen in liquid nitrogen and sectioned using a rotary cryostat (CM1850, Leica, Germany). Slides were

washed in PBS to remove excess OCT compound, stained with Alexa Fluor®488 Phalloidin (Invitrogen) and mounted in Vectashield Mounting Media with DAPI (Vector Laboratories Ltd, Peterborough, UK). Observation under light and fluorescent microscopy and digital image acquisition was carried out with an inverted microscope (Olympus IX 71).

2.6 Statistics

Results are presented as mean $\pm$ standard deviation. A students T-test was used to test significance between specific cases, results were considered significantly different at $p < 0.05$. For each group tested as described above, the mechanical tests comprised 5 specimens divided into two ring sections tested for each sample resulting in an effective $n=10$ for each group.

3. Results

3.1 Decellularization

Histological examination of both native arterial tissue and tissue subjected to the decellularization process is shown in Fig 1. H&E staining of the native tissue displayed the characteristic medial lamellar units of SMCs surrounded by collagen fibers within concentric layers of elastin (ECM stains pink, cells black). Decellularized tissue confirmed complete removal of native vascular cells with a remaining intact porous matrix of multiple concentric layers of collagen and elastin (Fig 1 A-B). The wall thickness decreases with the removal of cells. Masson's Trichrome stain further verified cell removal by the absence of stained cytoplasm in the decellularized tissue (cytoplasm stains pink and collagen blue). The morphology of the matrix is more visible with this collagen specific stain, the bundles of collagen fibers are seen tightly packed in the native tissue. The absence of SMCs in the decellularized tissue allows the collagen fibers to loosen but still maintain their circumferential orientation (Fig 1 C-D). Closer examination of the collagen after decellularization by Picrosirius Red staining showed the presence of smaller collagen fibers (collagen stains red) not visible with the Masson's Trichrome stain. The native tissue once again displayed the tight packing of the collagenous ECM (Fig 1 E-F). The pores created by the removal of the cells are seen to contain small collagen fibers resulting in a porous but highly dense matrix after decellularization. DAPI staining was used to further verify the removal all cells and cell remnants. The circumferential alignment and orientation of the

SMCs nuclei was visible in the native tissue with no visible fluorescent material in the decellularized tissue (Fig 1 G-H).

3.2 Decellularized tissue with medial cavities

Three 300 μ m diameter Nitinol needles were successfully inserted into the wall of the native tissue prior to decellularization. None of the needles penetrated the vessel wall and were inserted at approximately 120° circumferentially to each other. The addition of the needles did not hinder the process of decellularization as can be seen in Fig 2. H & E staining confirmed the removal all cells from the tissue and this was additionally verified with DAPI staining (Fig 2 A-C). The ultrastructure of the tissue did not appear altered by the addition of the needles. The concentric layers of collagen and elastin were visible in the same configuration as the decellularized tissue without the needles (Fig 2 A). Three cavities or channels were evident at the locations of the inserted needles in the medial layer (Fig 2 B). These medial cavities were elliptical in shape with major axis of approximately 275 μ m and a minor axis of 125 μ m. The cavities or channels displayed minimal damage to the ECM components along the length of the tissue and maintained their open configuration upon needle removal. The needles were inserted within a lamellar unit and appeared to spread or widen this layer as they were inserted further down the length of the tissue. The cavity expanded the surrounding tissue without cutting or shearing any of the bundles of collagen fibers or elastin sheets, resulting in extremely localised and minimal matrix disruption (Fig 2 B).

3.3 Collagen digested tissue

Picrosirius Red staining of the four groups described above is shown in Fig 2 and reveals the highly dense nature of the collagen network in the native tissue. After decellularization a porous 3-D network of collagen remained. The pores created by the removal of the cells are intersected with small collagen fibers resulting in small pore sizes and tight packing of the large collagen fiber network and elastin layers, producing a tight, dense matrix (Fig 2 E). Collagen digested tissue subjected to 15 mins sonication in NaOH created a much more porous scaffold. A great deal of the small collagen fibers were digested and removed from the tissue which created a matrix with an obvious increase in pore size (Fig 2 F). Digestion of the large collagen fibers was not apparent and the overall collagen network appeared fully intact. Decellularized tissue with medial cavities subjected to 15 mins sonication in NaOH had a

similar increase in porosity as a result of the collagen digestion. The medial cavities created by the needles appeared identical to those in the decellularized tissue, causing minimal disruption to the surrounding ECM (Fig 2 G). The trend of increasing porosity with collagen digestion continued in the 90 mins sonication group. Small collagen fibers are almost entirely removed from these samples and a major increase in the overall porosity of the scaffold is apparent. The large collagen fibers appear slightly reduced in diameter and stained much weaker, however the overall collagen network remained intact (Fig 2 H). Large scale disruption was evident in the final group which were subjected to 120 mins sonication in NaOH and 24 h mechanical agitation. The remaining collagen in these samples stained very weakly and there was an obvious large scale reduction in the diameter of the collagen fiber network which was consistent across the scaffold wall (Fig 2 I). A resemblance to the collagen network remained within the tissue but was not intact. Rupture of the collagen fiber network was visible throughout the scaffold. This is particularly evident when the stained sections are viewed at a higher magnification. At numerous locations across the scaffold the disintegration of the collagen fibers network was noticeable with the unravelling of bundles of collagen and actual rupture of fibers as indicated with arrows (Fig 2 J).

3.4 Mechanical Testing

The stress-strain curves displayed a typical hyperelastic response of a biological soft tissue (Fung, 1993, Humphrey et al., 2002). A bi-phasic response due to the interaction between the collagen and elastin is observed which consists of a toe region, followed by an upturning transition region ending with a linear response (Fig 3 D). The elastin is responsible for the toe region of the graph relating to high strain levels for low stresses and is referred to herein as the 'elastin dominant phase'. A linear elastic response is visible in this elastin dominant phase corresponding to the first third of the stress-strain data. The slope of this third of the data was determined as the modulus of the elastin component of the tissue and was calculated for each specimen tested. As the stress increases the collagen begins to engage more readily until it bears the full load at higher stresses corresponding to the final linear region of the graph and is referred to herein as the 'collagen dominant phase'. The slope of this linear region, corresponding to the final third of the stress-strain data is the modulus of the collagen component of the material and was determined for each specimen tested.

The averaged stress-strain curves ($n=10$ each) are shown in Figs 4 and a summary of the mechanical properties can be seen in Table 1. Specimens subjected to decellularization

produced a similar overall mechanical response to that of the native tissue however, there were some noticeable differences in the decellularized group. The modulus of the elastin dominant phase was significantly higher with much less extension resulting in an earlier advent of the collagen region of the curve. The decellularized tissue failed at a much higher UTS, was significantly stiffer in the collagen dominant phase and also went to a higher strain at failure. The additional insertion of the needles prior to decellularization resulted in a further change to some of the mechanical properties while still maintaining an overall response similar to the native and decellularized tissue groups (Fig 5). The modulus of the collagen dominant phase was higher than the native tissue but was slightly decreased from that of the decellularized tissue. The modulus of the elastin dominant phase was hugely reduced and there was considerably more distension in this phase before the advent of the collagen dominant phase.

Digesting collagen from the decellularized tissue caused notable changes to the mechanical properties of the scaffold. After 15 mins sonication in NaOH the scaffold still displayed the bi-phasic mechanical response as seen in Fig 3 D. The distension of the elastin dominant phase was apparent with much higher strains noted at the transition region of the graph. A decrease in the modulus of the collagen dominant phase was seen compared to decellularized tissue. However the UTS is similar to that of the decellularized tissue. The additional creation of medial cavities combined with 15 mins sonication in NaOH showed a similar amount of distension in the elastin dominant phase to that of 15 mins sonication with a similar modulus in the elastin dominant phase. A similar pattern to the decellularized tissue with medial cavities was evident in the collagen dominant phase as a decrease in both modulus and UTS occurred with the creation of the medial cavities (Fig 5). Following 90 mins sonication in NaOH a very similar response to the 15 mins sonication group was seen; the main difference was a lower modulus in the collagen dominant phase, a lower UTS and higher strain at failure. The final group subjected to 120 mins sonication and 24 h agitation in NaOH displayed a significantly different response. The samples were visibly weaker and more translucent prior to the commencement of the tensile test. The modulus of the collagen dominant phase and UTS was extremely low. The modulus of the elastin dominant phase and strain at failure was similar to the other groups.

3.5 Scaffold Repopulation

The results of the cell injections are shown in Figure 6. Penetration of the non collagen digested decellularized tissue proved ineffective when injecting hSMCs through the medial cavities. Phalloidin stained section (cytoplasm stains green) shows cells were present within the medial cavity, filling up this channel, but were not visible within the tissue away from the injection site (Fig 6 A). This was consistent throughout the length of the injected scaffold. DAPI staining of the injected scaffolds sonicated for 15 mins in NaOH again displayed a filling of the medial cavity with cells, and in addition minimal infiltration of the surrounding tissue was also observed (Fig 6 B). Examination of the injected 90 mins sonicated scaffold revealed a much higher rate of cell infiltration away from the medial cavity (Fig 6 C). Cells were present outside of the medial cavities demonstrating increased circumferential dispersion compared to the other scaffolds. The higher cell concentration and longer seeding times of the 30 mm long scaffolds showed much higher levels of cell repopulation (Fig 6 D-E). Increased circumferential dispersion of cells away from the medial cavity is clear with large cell numbers retained after 24 h of static culture. These cells are located across a number of elastin layers and extend circumferentially in both directions away from the injection site, a high magnification view of the DAPI stained section shows that the cells have infiltrated between a number of layers of elastin (Fig 6 F).

4. Discussion

One of the remaining goals of vascular tissue engineering is the realisation of a fully populated contractile SMC medial layer within realistic culture times. If this goal can be achieved the probability of translating the technology to clinical application will be significantly improved. Attempts have been made to overcome this problem within synthetic scaffolds for vascular applications using rotational vacuum techniques (Soletti et al., 2006, Nieponice et al., 2008, Kasyanov et al., 2009, Godbey et al., 2004), the high porosity and large pore size make these scaffolds easier to seed with large cell numbers in short time periods. While in the case of dense decellularized tissue bulk seeding the medial layer has not been successfully achieved. This research aimed to address this by customizing the properties of decellularized porcine carotid arteries by creating a method for bulk seeding cells into the media while not sacrificing mechanical integrity.

The dense architecture makes injection of cells within the wall of decellularized scaffolds unfeasible (Yazdani et al., 2009). To create a method of bulk seeding the medial layer of this

decellularized tissue, we inserted needles longitudinally into the medial layer of native tissue prior to the decellularization process. This created a scaffold that contains cavities or channels within the media when the needles are removed. Removing these needles and simultaneously injecting cells through them allows the channels to be filled with SMCs hence assisting the bulk cell seeding of the scaffold. The needles and subsequent channels created upon their removal essentially allow a path for entry of cells to the medial layer. Cells will still have to migrate throughout the rest of the scaffold from within these channels. However, the issue of the extremely dense matrix which hinders cell infiltration still exists. The needles result in limited localised disruption to the ECM but the surrounding area will still impede scaffold repopulation. In an effort to overcome this, decellularized tissue was subjected to a collagen digestion protocol to increase the porosity of the scaffold but still maintain scaffold integrity.

There have been previous attempts to increase the porosity in decellularized tissue (Bergmeister et al., 2005). Axial perforation of the decellularized tissue with 50 μ m holes using a laser noted a higher level of cell repopulation *in vivo*. Pure collagen and pure elastin scaffolds have been studied (Chuang et al., 2009, Simionescu et al., 2006, Lu et al., 2004, Kurane et al., 2007). Completely removing these individual ECM components noted a higher infiltration of cells in the elastin scaffolds *in-vivo*. Subdermal implantation of these scaffolds not only showed increased host cell infiltration but demonstrated new ECM synthesis within the scaffold (Simionescu et al., 2006). The benefits of increasing the porosity to aid cell infiltration are clear but require a fine balancing act between removing ECM components and not adversely affecting the mechanical properties of the scaffold. Although both collagen and elastin contribute to the mechanical response of the tissue (Holzapfel, 2008) we chose to digest collagen. The more complex elastin network is difficult to synthesize from scratch and a lack of elastin is a leading cause of graft failure *in vivo* due to compliance mismatch (Greenwald and Berry, 2000, Patel et al., 2006, Lee et al., 2011). A scaffold without an elastin network would not provide the elastic response necessary to match a native artery and is therefore essential for any vascular graft to have a fully matured elastin network (Lee et al., 2011, Fonck et al., 2007).

Small collagen fibers were most notably affected by the NaOH protocol resulting in a progressively more porous scaffold in response to duration of sonication. Sonication for 90 mins proved the most efficient sonication duration to remove the majority of the small

collagen fibers while not affecting the collagen fiber bundle network. Excessive digestion in NaOH for 120 mins sonication and 24 h agitation resulted in the breakdown of this collagen fiber bundle network displaying widespread tissue disruption. These results showed that successful digestion of collagen fibers in a controlled manner can be utilised to modify or tailor the collagen density of the scaffold.

Mechanical testing of these manipulated scaffolds was carried out to determine the effect on the overall mechanical response of the tissues. Rectangular or dumb-bell shaped specimens cut from the opened strips of tissue produce different mechanical responses in both the axial and circumferential directions (Amiel et al., 2006, Funamoto et al., 2010). Cutting the arterial wall open releases residual stresses, particularly from the elastin network. Specimens with a disrupted elastin network do not truly reflect the interaction of the collagen and elastin within the arterial wall when tested (McFetridge et al., 2004, Seliktar et al., 2000, Venkatasubramanian et al., 2006). We opted to keep the arterial wall intact and tensile tested 4mm long ring sections to failure.

Decellularizing the native arterial tissue produced a stiffer less distensible tissue which matches the results obtained elsewhere in the literature (Williams et al., 2009, Roy et al., 2005, Conklin et al., 2002). The stiffening of the tissue and reduction of the elastin dominant phase of the stress-strain curve is attributed to the loosening of the tissue due to cell removal, and uncrimping of collagen fibers. This causes an earlier than normal engagement of the collagen fibers in the direction of the applied strain (Williams et al., 2009) hence the early upturn in the transition region of the graph and the increase in modulus of the collagen dominant phase. This increased fiber mobility is due to the increased porosity and less compacted nature of the decellularized tissue resulting in easier recruitment and reorientation in the direction of the applied strain of the collagen fibers.

The creation of medial cavities within the tissue also produced a stiffer tissue; whereby the modulus of the collagen dominant phase is significantly higher than the native tissue. This can again be accounted for by the loosening and uncrimping of the collagen fibers. The reduction in modulus from the decellularized tissue may be accounted for by the local deformation created by the needle insertion delaying full collagen recruitment in this final linear region. The increase in distensibility in the elastin dominant phase is combined with a significant decrease in the modulus compared to the decellularized tissue. This noticeable

change in the response of the tissue must be as a result of the needle insertion. Two possible explanations for this are; (i) the localised disruption created by the medial cavities may hinder collagen fiber recruitment resulting in a slight delay in collagen fiber engagement hence extending the elastin dominant phase, (ii) the needle disruption affects elastin struts and a small number of elastin sheets locally reducing the effectiveness of the elastin network and also reducing the elastin dominant phase stiffness, hence delaying the transfer of the load to the collagen fibers. It is important to note that while these results signify disruption to the ECM, the overall function of the elastin remains intact and combines with the collagen network to produce a comparable mechanical response to the native tissue.

Customisation of the tissue by collagen digestion demonstrated that the tensile response of manipulated decellularized tissue can be tuned to closely mimic the response of native tissue. The digestion of the small collagen fibers and not the collagen fiber bundles ensures that the tissue maintains sufficient UTS, and mirrors the stiffness of native tissue. The collagen digestion protocol that delivers potentially the optimum result involves NaOH digestion and 90 mins sonication. This protocol ensures that the elastin and collagen region stiffness's match the native tissue and the overall strength of the tissue is not compromised. The addition of medial cavities to this experimental protocol reduces the stiffness and the strength, demonstrating that some damage has been initiated in the ECM components. However this damage is not sufficient to cause significant adverse effects on the tensile response of the tissue. The strain to failure increases in all the collagen digested groups and we speculate that the mechanism for this is due to the removal of the small collagen fibers this leads to fewer uncrimped fibers available for early recruitment which delays the collagen engagement and load transfer from the elastin. The final group subjected to 120 mins sonication and 24 h agitation in NaOH displayed a markedly different response (Fig 4) indicating that the collagen fiber bundle network was significantly affected by the NaOH digestion, supported by the histology data showing complete fiber breakdown (Fig 6G). The elastin dominant phase modulus and strain at failure was similar to the other groups signifying that the elastin structure remains undisrupted by the collagen digestion. This demonstrates that only collagen is affected by our customisation techniques suggesting explanation (ii) above is unlikely to be responsible for the noted changes in mechanical properties. Relatively high standard deviations were observed in the mechanical test results, particularly within the collagen digested groups. These standard deviations may be attributed to the varying collagen content of tested samples due to the inter specimen variations within

the animals from which the tissue was harvested. While these deviations are large the assertions made regarding the overall mechanical response of the tested groups are unaffected by them.

The scaffold repopulation results indicate that the medial cavities are an ideal method of repopulating the medial layer with hSMCs. The higher cell concentration of 1×10^6 cells per needle injected within the 90 mins sonicated scaffold demonstrated optimum results (Fig 6 D-E). Poor cell infiltration is observed within the undigested and 15 mins sonicated scaffolds with the majority of cells filling the medial cavity and not the media of the scaffold. However successful circumferential distribution of cells within the 90 mins sonicated scaffolds was observed. This seeding technique is quicker and more effective at repopulating the deep medial layer than the most commonly used static cell seeding methods published in the literature (Neff et al., 2010, Villalona et al., 2010, Yazdani et al., 2009). Further optimisation of the injection protocol is required in order to produce consistent fully repopulated scaffolds.

5. Conclusions

We demonstrated that decellularizing and customising porcine arterial tissue can result in a novel scaffold with adequate strength, compliance, porosity and a built-in route to repopulate the medial layer. The formation of medial cavities running the length of the scaffold created an ideal access route for seeding SMCs. Digesting collagen increased the scaffold porosity. We demonstrated that customising decellularized tissue in this manner was shown to cause minimal local disruption to the ECM components and does not have a detrimental effect on the mechanical response of the tissue. An initial cell injection study through the medial cavities demonstrated the feasibility of this approach, whereby the most porous scaffold demonstrated the highest level of cell infiltration. Future studies will involve bulk seeding this scaffold and its maturation in a bioreactor.

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Figure 1 Histology of porcine carotid arterial tissue before (A,C,E,G) and after (B, D, F, H) decellularization. (A) H&E stained native artery showing cell configuration and structure of ECM components. (B) H&E stained decellularized tissue showing removal of cells and remaining porous ECM. (C) Masson's Trichrome stain showing high cell density embedded in collagenous ECM. (D) Complete cell removal and remaining undisrupted collagen fiber network. (E) Picrosirius Red stain of native tissue shows dense collagen content. (F) Picrosirius Red stain after decellularization showing a porous collagen structure. (G) DAPI stain shows highly cellular native tissue. (H) After decellularization DAPI reveals no cells or cell remnants.

Figure 2 Picrosirius Red stain showing collagen digestion of decellularized porcine carotid arteries. (A) Native tissue displaying highly dense collagenous ECM. (B) Decellularization reveals a porous architecture and dense collagen network. (C) 15 mins sonication in NaOH showing increased porosity with digestion and removal of small collagen fibers evident. (D) Creation of medial cavities does not affect mechanism of collagen digestion (E) 90 mins sonication in NaOH shows removal of all small collagen fibers with the collagen fiber bundle network intact. (F) Samples digested with 120 mins sonication & 24 h agitation in NaOH showed weak staining and a disrupted collagen fiber bundle network. (G) High magnification shows a disrupted collagen network and breakdown of collagen fiber bundles after 120 mins sonication and 24 h agitation in NaOH, arrows indicate collagen fiber bundle rupture. (H) H&E stain of entire scaffold section showing ECM structure and three medial cavities. (I) Higher magnification H&E stain of an individual medial cavity showing an elliptical structure with minimal localized ECM disruption and separation of elastin sheets. (J) DAPI stained section of medical cavity showing no affect on decellularization process due to creation of medial cavity.

Figure 3 Mechanical testing of scaffold. Bulk view native tissue with three micro-needles inserted longitudinally into the proximal wall. (A) Schematic of mechanical ring test setup showing specimen and marked dots for video extensometer tracking. (B) Example of tested specimen showing extension of tissue in response to the applied load ending in specimen failure (i-v). (C) Typical stress-strain response of arterial tissue. Sample response of a tested specimen with linear responses fitted to the first and last 30% of the curve marking the elastin dominant phase and collagen dominant phase respectively.

Figure 4. Stress-strain curves of native porcine tissue, decellularized tissue and decellularized tissue with medial cavities. (A) Similar mechanical response is seen in each tissue group. The decellularized tissue is stiffer and less distensible than the native tissue. The creation of medial cavities produces a stiffer tissue than the native but less stiff than the decellularized tissue and has an increase in distensibility. (B) Stress-strain curves of collagen digested decellularized tissue. The collagen digested tissue is more distensible than the native tissue. A similar mechanical response is seen after 15 mins and 90 mins sonication. The creation of medial cavities did not affect the tissue response. Excessive collagen digestion grossly altered the tissue response.

Figure 5 Comparison of mechanical properties of all tested groups. (A) Modulus of the collagen dominant phase increases after decellularization and decreases with collagen digestion. A drop in modulus is seen with creation of medial cavities. (B) Elastin dominant phase varies significantly after decellularization and is reduced by medial cavity creation and

collagen digestion, returning the modulus value to that of the native tissue. The levelling off of the modulus of the elastin dominant phase after scaffold customization indicates that the elastin network is fully functioning and undisturbed. (A) Strain at failure is seen to increase after decellularization due to cell removal and increased collagen fiber mobility and uncrimping of collagen fibers. A further increase is seen after further scaffold customization due to medial cavity creation and collagen digestion. (B) UTS increases significantly after decellularization and a reduction is seen with medial cavity creation. 15 mins sonication maintains the same UTS but decreases with further collagen digestion, with a dramatic reduction after 12 mins sonication & 24 h agitation.

Figure 6 Scaffold repopulation with hSMCs by direct injection through medial cavities. (A) 10mm long decellularized scaffold injected with 500,000 hSMCs displays no infiltration outside of the medial cavity. (B) DAPI stained section of a scaffold digested by 15 mins sonication to increase porosity shows some cell infiltration away from the medial cavity. (C) Large cell infiltration away from the site of injection is seen after 90 mins sonication. (D) Phalloidin stain of a 90 mins sonicated 30mm long scaffold injected with 1×10^6 hSMC per medial cavity displayed a much greater increase in the circumferential dispersal of cells away from the injection site. (E) Each medial cavity on the 90 mins sonicated scaffold displayed similar successful repopulation away from the medial cavity. (F) High magnification of a DAPI stained section reveals cells infiltrate between elastin layers.

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Table 1. Mechanical properties of native and decellularized porcine carotid arteries.

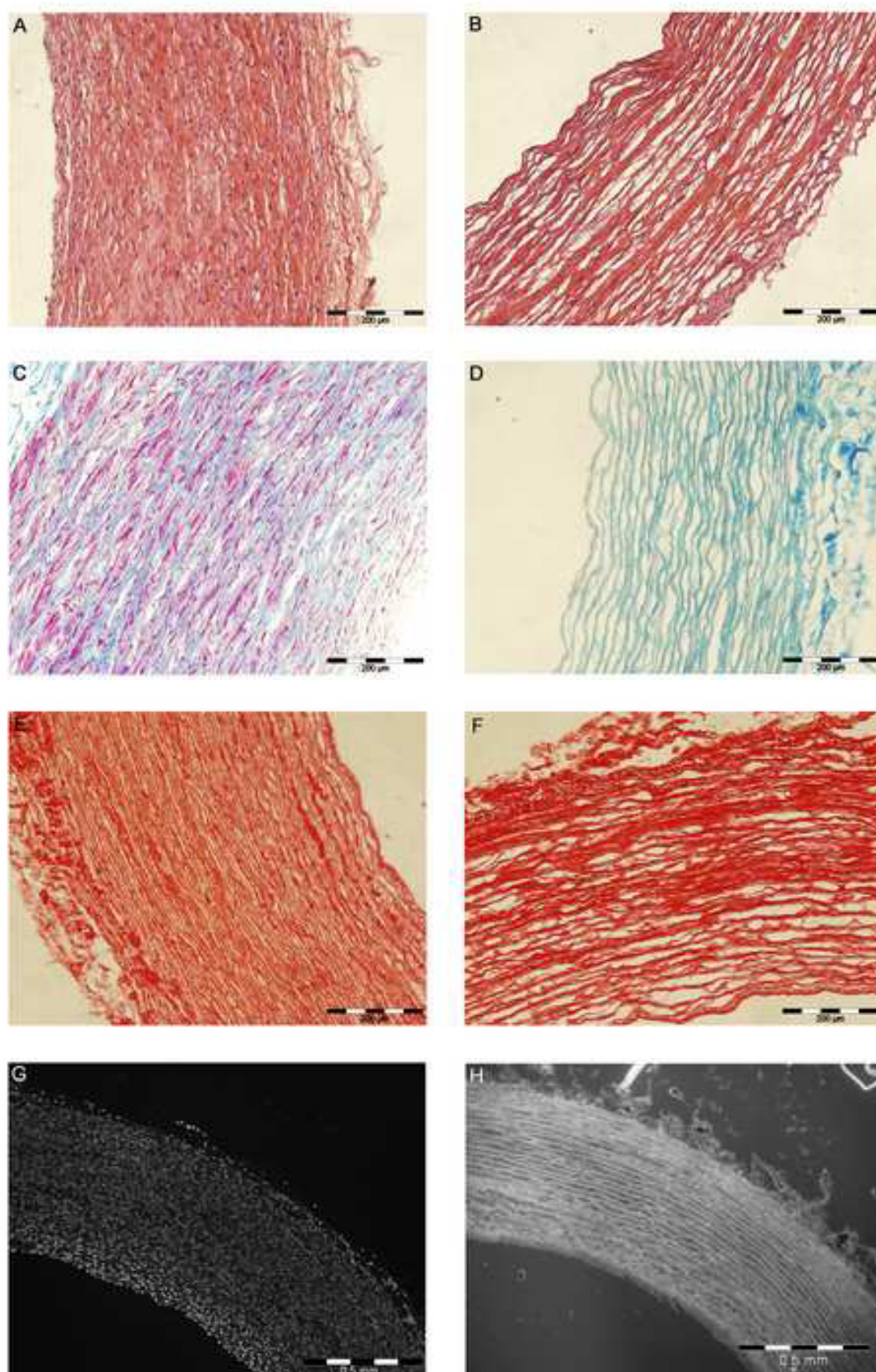
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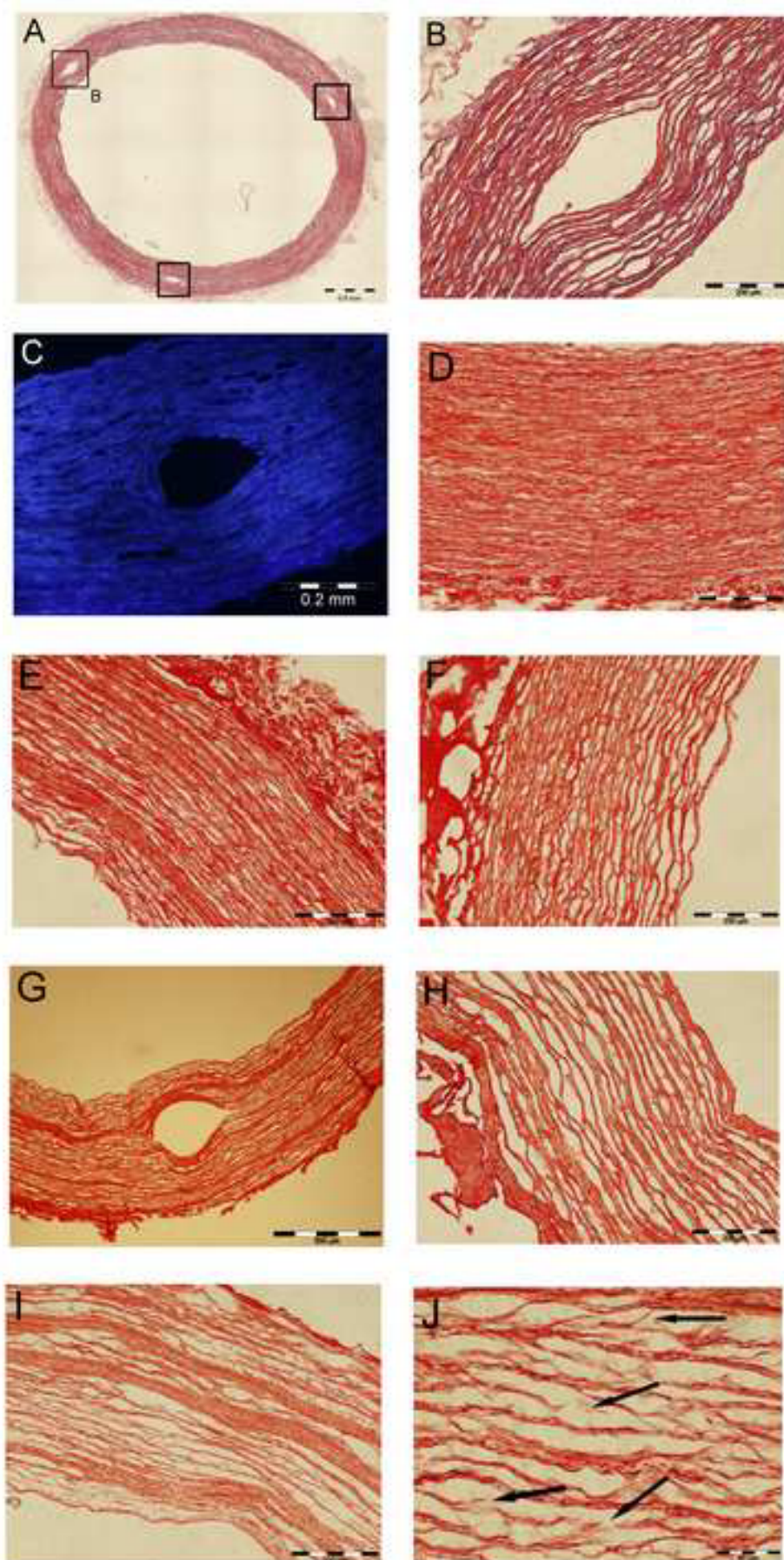
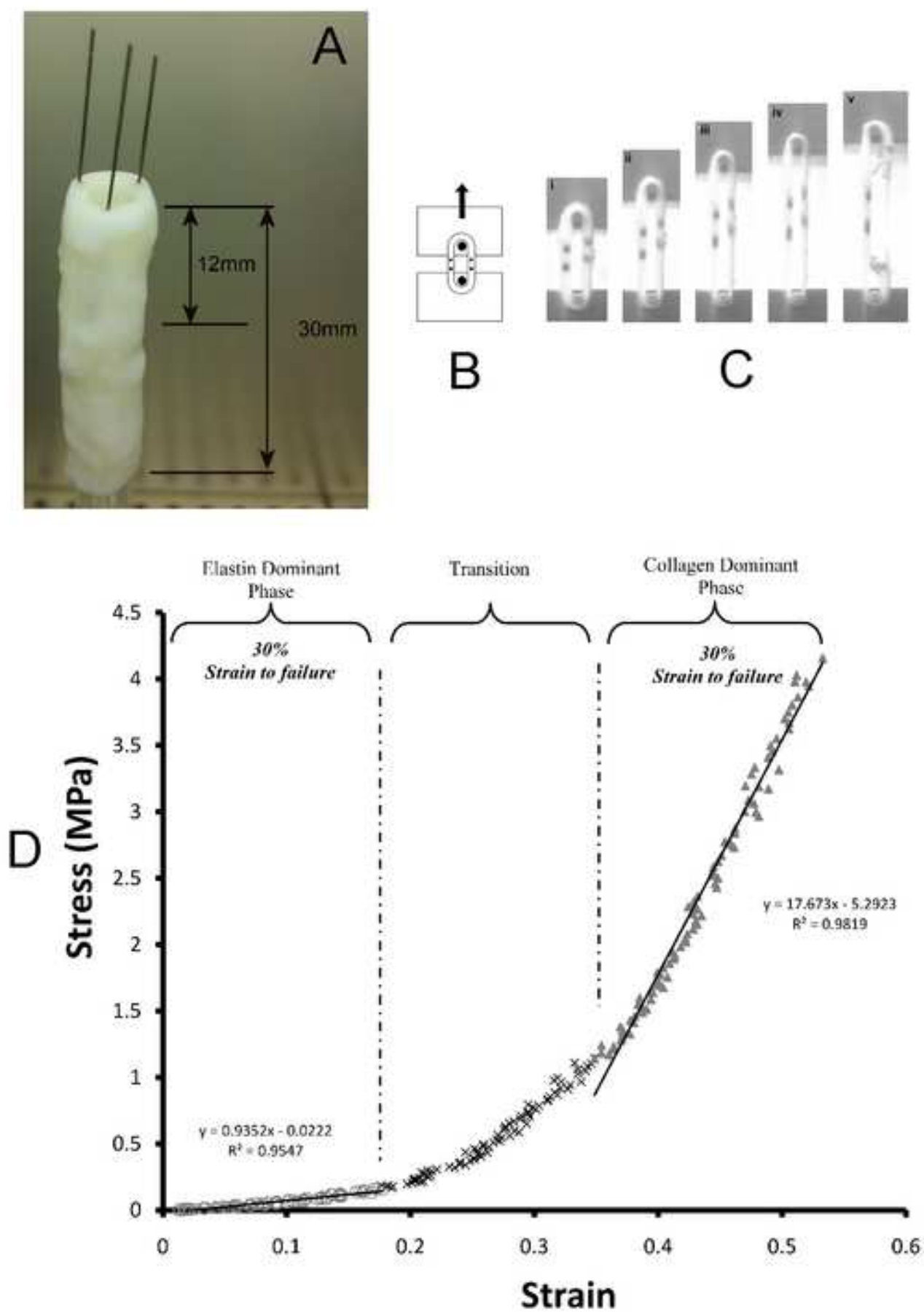
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Figure 3

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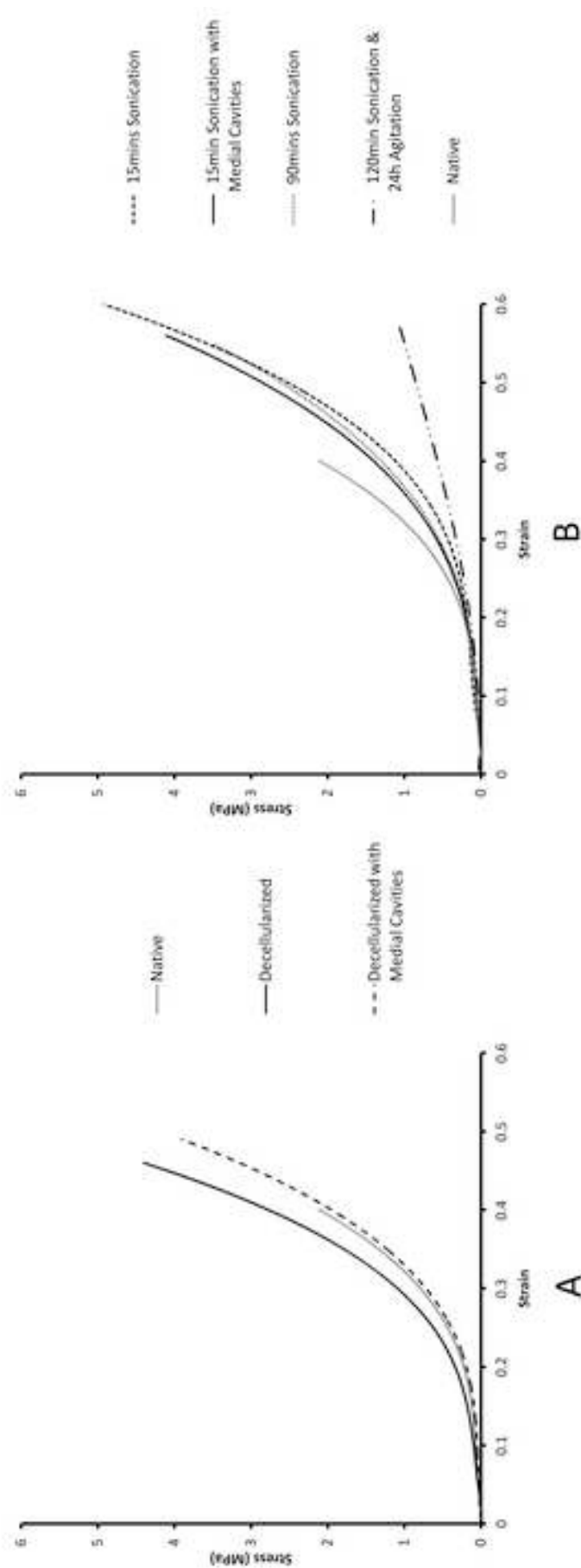


Figure 4
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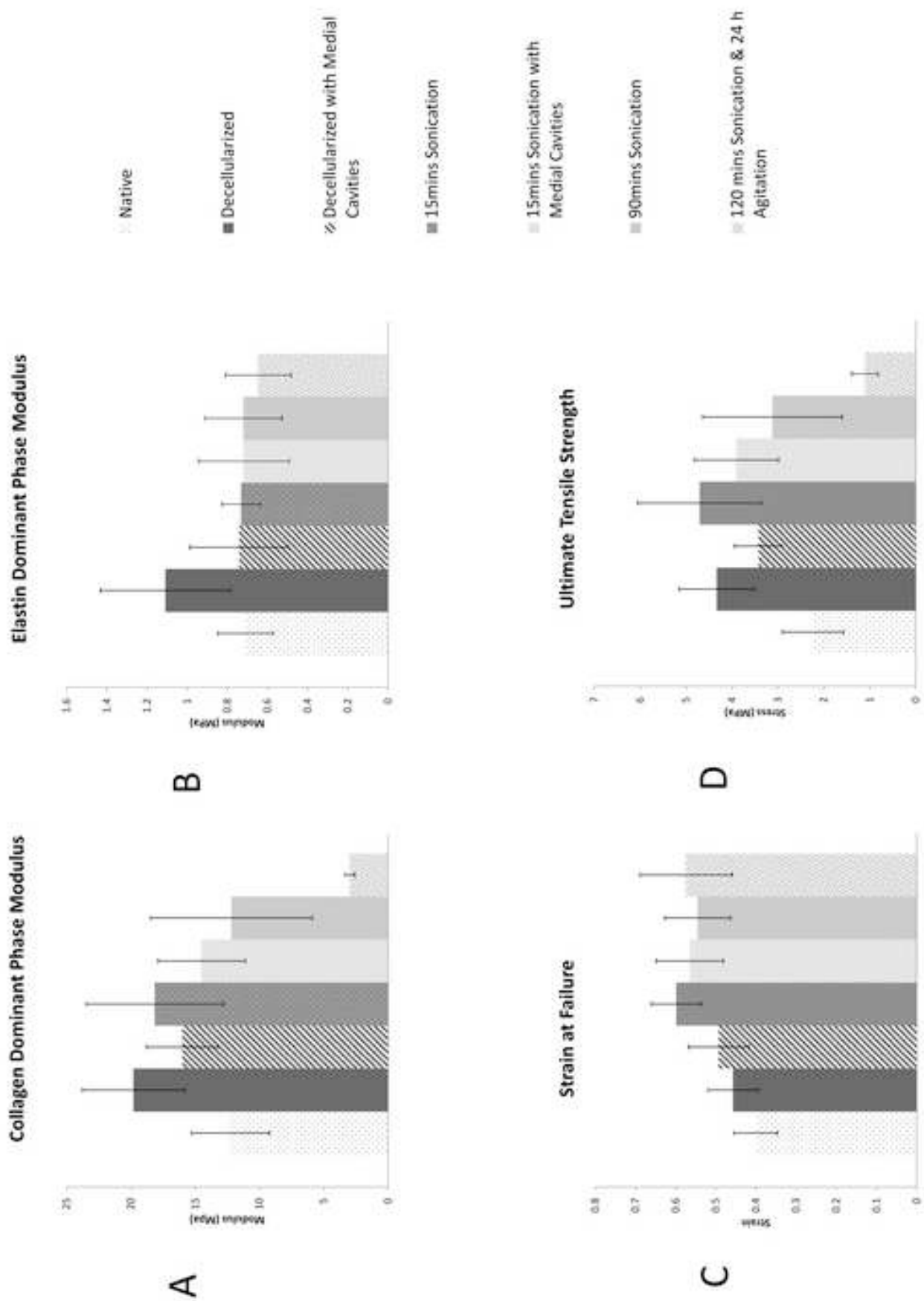


Figure 5
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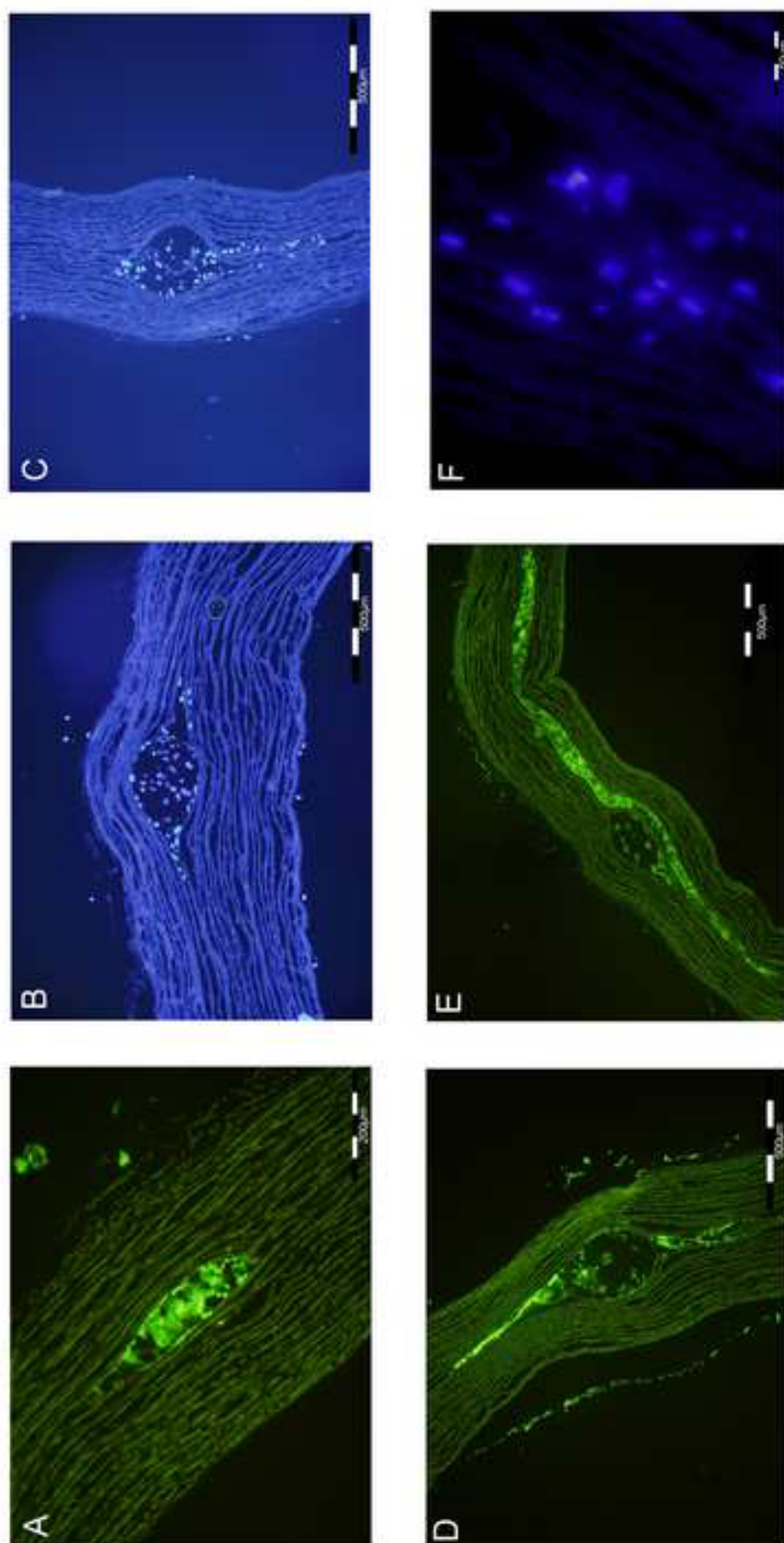


Figure 6

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Table 1
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	Elastin Dominant Phase Modulus (MPa)	Collagen Dominant Phase Modulus (MPa)	Strain at Failure	UTS (MPa)
Native	0.71 ± 0.14 ^b	12.26 ± 3.04	0.40 ± 0.05	2.24 ± 0.67
Decellularized	1.11 ± 0.32	19.81 ± 3.97 ^a	0.46 ± 0.06	4.34 ± 0.82 ^a
Decellularized with MC	0.74 ± 0.24 ^b	16 ± 2.78 ^a	0.49 ± 0.08 ^a	3.43 ± 0.52 ^{a,b}
15mins Sonication	0.73 ± 0.1 ^b	18.15 ± 5.35 ^a	0.6 ± 0.06 ^{a,b}	4.71 ± 1.35 ^a
15mins Sonication with MC	0.72 ± 0.23 ^b	14.50 ± 3.4	0.57 ± 0.08 ^a	3.91 ± 0.92 ^{a,b}
90mins Sonication	0.72 ± 0.19 ^b	12.19 ± 6.3 ^b	0.55 ± 0.08 ^{a,b}	3.13 ± 1.52 ^b
120mins Sonication & 24h Agitation	0.65 ± 0.16 ^b	2.97 ± 0.38 ^{a,b}	0.57 ± 0.11 ^{a,b}	1.11 ± 0.29 ^{a,b}

^a Significant difference from native

^b Significant difference from decellularized

MC = Medial Cavities