

INVESTIGATION OF SMALL-DIAMETER DECELLULARISED ARTERY AS A POTENTIAL SCAFFOLD FOR VASCULAR TISSUE ENGINEERING

A dissertation submitted for the degree of Ph.D.

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D. is entirely my own work, and that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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Publications Resulting from this Study

Peer Reviewed Papers

E.M. Campbell, P.A. Cahill and C. Lally. Investigation of a small-diameter decellularised artery as a potential scaffold for vascular tissue engineering; Biomechanical evaluation and preliminary cell seeding. *Journal of the Mechanical Behavior of Biomedical Materials*, 2012; 14:130-142

Peer Reviewed Conference Abstracts

E.M. Campbell, P.A. Cahill and C. Lally. Repopulation and biomechanical evaluation of a small-diameter decellularised porcine coronary artery scaffold; a potential vascular graft. In *Proceedings of the Nineteenth Annual Conference of the Section of Bioengineering of the Royal Academy of Medicine in Ireland*, Meath, Ireland, p.73, 2013 [Presented January 19th, 2013, Meath, Ireland]

E.M. Campbell, J.N. Mackle, P. Gatenholm and C. Lally. Strain induced remodelling of potential scaffolds for tissue engineered blood vessels. *Tissue Engineering and Regenerative Medicine International Society – EU Chapter Meeting*, Granada, Spain, 2011 [Presented June 9th, 2011, Granada, Spain]

E.M. Campbell and C. Lally. Optimum biomechanical behaviour of vascular scaffolds determined using short-term decellularisation of porcine coronary arteries.

In *Proceedings of the 17th Congress of the European Society of Biomechanics*, Edinburgh, Scotland, 2010 [Presented July 5th, 2010, Edinburgh, Scotland]

E.M. Campbell, J.N. Mackle and C. Lally. Cell seeding of decellularised porcine coronary arteries; a potential tissue engineered blood vessel. *Tissue Engineering and Regenerative Medicine International Society – EU Chapter Meeting*, Galway, Ireland, 2010 [Presented June 15th, 2010, Galway, Ireland]

E. Campbell and C. Lally. Biomechanical properties of fresh and decellularised porcine coronary arteries; insights into the optimum tissue engineered blood vessel. *3rd Marie Curie Cutting Edge Practical Training Course: Tissue engineering, stem cells and biocompatibility testing of biomaterials*, Aachen, Germany, 2009 [Presented August 28th, 2009, Aachen, Germany]

E. Campbell and C. Lally. Mechanical characterisation of the natural matrix scaffold in coronary arteries; a potential scaffold for tissue engineering. In *Proceedings of the 15th Annual Conference of the Section of Bioengineering of the Royal Academy of Medicine in Ireland*, Limerick, Ireland, p.48, 2009 [Presented January 31st, 2009, Limerick, Ireland]

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Nomenclature

N ₀	Non-decellularised porcine coronary artery
N ₉	9 hour non-decellularised porcine coronary artery
N ₃₀	Non-decellularised porcine coronary artery cultured for 30 days
D ₉	9 hour decellularised porcine coronary artery
D ₁₅	15 hour decellularised porcine coronary artery
D ₃₀	Decellularised porcine coronary artery cultured for 30 days
S ₃₀	Cell-seeded decellularised porcine coronary artery cultured for 30 days

Abstract

Investigation of small-diameter decellularised artery as a potential scaffold for vascular tissue engineering

Evelyn M. Campbell

The successful replacement of small-diameter blood vessels, affected by cardiovascular disease, with natural and synthetic bypass grafts remains limited due to long-term patency issues. The development of a small-diameter tissue engineered blood vessel (TEBV), with equivalent biomechanical properties to the vessel being replaced, may provide a potential solution. This thesis aims to determine the biomechanical properties of porcine coronary arteries (PCA) and the corresponding natural matrix scaffold of the artery through short-term decellularisation providing vital information for the development of an optimum TEBV, while also establishing the potential of this natural matrix scaffold to be used as a vascular tissue engineering scaffold.

The natural matrix scaffold of small-diameter (< 4 mm) PCA was achieved through a short-term decellularisation method to limit the natural degradation of the tissue. The biomechanical properties of PCA and the corresponding natural matrix scaffold of the artery were established. Tubular segments of PCA, up to 60 mm in length, were perfused with 0.1 M sodium hydroxide for 6 to 12 hours to achieve the natural matrix scaffold. Uniaxial tensile and inflation tests confirmed the scaffold maintains its non-linear response, however a less stiff, more distensible low-load response and stiffer high-load response was found compared to non-decellularised sections. Vascular smooth muscle cells were successfully seeded to the luminal and abluminal surfaces and lateral edges of decellularised sections and attachment and infiltration of the xenogeneic cells after 15 days confirmed the viability of the scaffold as a suitable

environment for cell growth and infiltration. The maintenance of the internal elastic lamina provided a suitable surface for the attachment of endothelial cells. Biomechanical tests of the cell-infiltrated scaffold confirmed a recovery of the native artery distensibility. Additionally, the bare scaffold remained viable for 30 days under pulsatile flow at physiological pressures confirming its potential as a TEBV scaffold.

Chapter 1 Introduction

1.1 Cardiovascular Disease

Cardiovascular disease (CVD) remains the principal cause of mortality and morbidity worldwide accounting for an estimated 17.3 million deaths or 30% globally in 2008 [1]. This figure is projected to reach 26.3 million by 2030. In Ireland CVD is the principal cause of death for both men and women, however, the percentage of deaths has fallen from a peak of 51% in 1980, to 41% in 1999, and now 32.9% in 2011 [2,3], see Figure 1.1. This reduction can be attributed to a number of factors including increased public health awareness, earlier access and treatment of acute cardiovascular events such as heart attack and stroke, surgical advancements, and continuing development of medical devices. The treatment of CVD results in a major economic burden accounting for an estimated €192 billion in the European Union in 2008 [4] and an estimated \$286 billion in the United States in 2011 [5]. These figures are a combination of direct costs including hospitalisation, medication, and physician costs, and indirect costs accounting for production losses due to morbidity and mortality.

The most prevalent form of CVD is coronary artery disease (CAD) accounting for an estimated 7.3 million deaths globally in 2008 [1]. A build-up of atherosclerotic plaque in the inner wall of the arteries supplying the myocardium results in a stiffer vessel and narrowed lumen. Depending on the severity of the stenosis, current treatments to revascularise a stenosed artery include drug therapy, angioplasty,

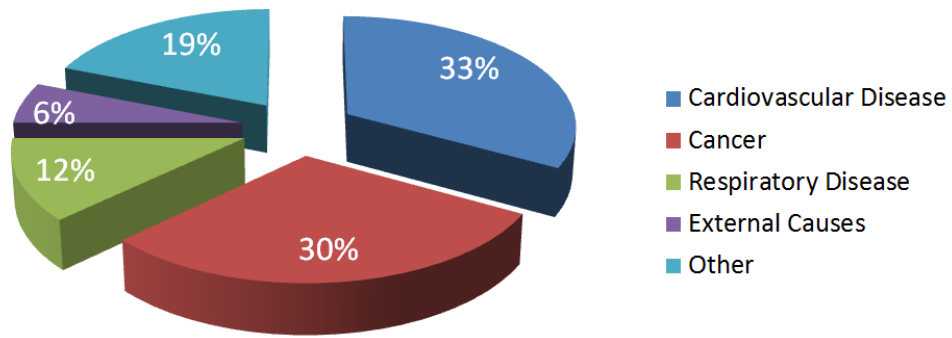


Figure 1.1: Principal causes of death in Ireland in 2011 [2].

stenting, and coronary artery bypass grafting (CABG). In 2009, 163,149 CABG procedures were performed in the United States [5]. Autologous blood vessel grafts or synthetic grafts are successfully used to bypass medium- and large-diameter diseased vessels (> 6 mm) [6]. However, for small-diameter vessels such as the coronary arteries, a mismatch between the biomechanical properties of both natural and synthetic grafts and the native vessel can compromise the long-term patency of the bypass grafts making them less than ideal substitutes [7,8]. This is demonstrated in Figure 1.2 where the mechanical property of compliance of various bypass grafts used in the femoral-popliteal position is compared. There is a clear correlation between decreased compliance in relation to that of the host vessel and a decrease in

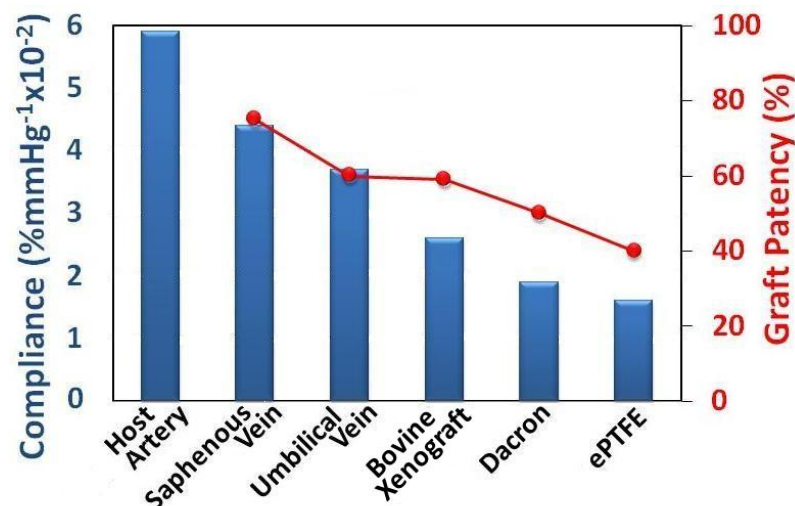


Figure 1.2: Biomechanical mismatch of current bypass grafts compared to the host artery; and the patency rates of the bypass grafts (adapted from [7]).

patency rates. This mismatch can lead to flow instabilities, thrombus formation, and intimal hyperplasia resulting in long-term graft failure [9]. The development of a small-diameter graft with corresponding biomechanical properties may overcome these limitations and improve the long-term patency rates for small-diameter applications such as CABG.

1.2 Objectives

Despite all the research efforts to date, there is still an unmet clinical need for small diameter vascular grafts or tissue engineered blood vessels (TEBVs) – extracellular matrix (ECM) based constructs present an exciting alternative because of their innate biomechanical properties. Clearly, the development of bypass grafts from coronary arteries would be highly advantageous as the vessel size and biomechanical properties would match that of the host vessel. In an effort to produce ECM-based TEBVs that match the structural, chemical, and biomechanical properties of native vessels, allogeneic and xenogeneic blood vessels have been decellularised providing a scaffold for the migration and growth of native endothelial cells and smooth muscle cells [10,11]. The ECM, consisting of structural and functional proteins, remains once the vascular cells have been removed. Due to the small size of coronary vessels, their numerous branches, and their limited accessibility no study has been carried out to-date to examine decellularisation of human or porcine coronary arteries and the resulting effect on their biomechanical properties.

For complete decellularisation, the ECM must be appropriately disrupted to allow for access to all cells and to provide a path for their subsequent removal. This disruption needs to be minimized in order to maintain native ECM biomechanical and biological properties [12]. A combination of chemical, enzymatic, and physical treatments have been used with treatment times ranging from 4 hours to several weeks. However, biomechanical degradation will occur simultaneously in these samples once removed from their native environment. Consequently, it is critical to establish a short-term decellularisation method to retain the native biomechanical properties as much as possible.

Additionally, a comprehensive understanding of the biomechanical properties of native blood vessels will aid in the development of comparative TEBVs. CABG is the most common bypass surgery performed, however, test data on the biomechanical properties of the coronary arteries is limited [13]. A thorough examination of the biomechanical properties and permeability of non-decellularised coronary arteries and the corresponding natural matrix scaffold is therefore necessary as it would provide vital insights into the ideal biomechanical properties for TEBVs and TEBV scaffolds.

To determine its potential as a TEBV scaffold the cell attachment capabilities of the natural matrix scaffold must be ascertained. A non-thrombogenic lining of the lumen of the scaffold which can support endothelial cells is required. The porosity of the scaffold should be adequate for vascular smooth muscle cell infiltration while withstanding physiological blood flow. Biomechanical analysis of the repopulated natural matrix scaffold will establish its suitability as a TEBV and determine the effect of the seeded cells in comparison to the biomechanical properties of non-decellularised and decellularised vessels.

A functional small-diameter TEBV graft which is biomechanically and biologically compatible with host vessels could improve long-term graft patency rates. Furthermore, it could provide suitable vessels for in vitro studies of vascular biology and the mechanisms of vascular disease and for pre-clinical tests of pharmacological and interventional treatments [14]. In order to achieve these goals the following objectives need to be realised, see also Figure 1.3:

1. Decellularisation:

- Development of a short-term decellularisation protocol to obtain tubular sections of the natural matrix scaffold of small-diameter porcine coronary arteries (PCA) established and verified by histological and immunocytochemical analysis.

2. Biomechanical Evaluation:

- Characterisation of the biomechanical properties and permeability of non-decellularised PCA and the corresponding natural matrix scaffold using a range of biomechanical tests and analysis.

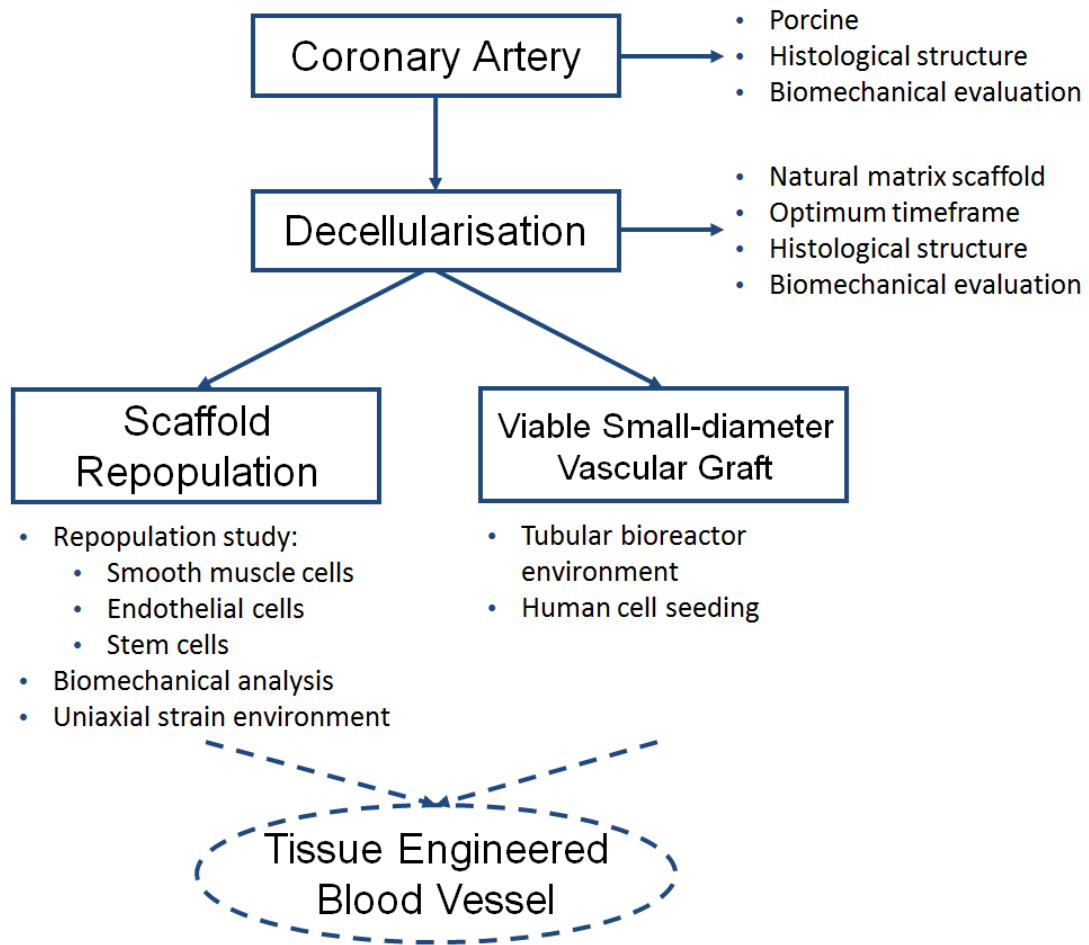


Figure 1.3: Flowchart outlining the major project objectives.

3. Scaffold Repopulation

- Determination of the suitability of the natural matrix scaffold of PCA as an environment for the growth of smooth muscle, endothelial, and stem cells.
- Characterisation of the proliferation, migration, and infiltration of repopulated cells in the scaffold under static and dynamic test conditions.
- Evaluation of the biomechanical properties of the repopulated scaffold.

4. Vascular Graft

- Determination of the effect of physiological pulsatile flow on decellularised PCA.
- Validation of the scaffold for human use by the seeding of human cells.

Chapter 2 Literature Review

2.1 Coronary Arteries

The cardiovascular system consists of the heart, the systemic circulation, and the pulmonary circulation. The coronary circulation, part of the systemic circulation, supplies oxygenated blood to the myocardium of the heart. The coronary arteries are the only branches of the ascending aorta, originating in the left and right aortic sinuses directly above the aortic valve [15]. Located just below the epicardial membrane in the coronary and interventricular sulci, the right coronary artery (RCA) supplies oxygenated blood to the right atrium and ventricle and the left main coronary artery supplies the left atrium and ventricle, see Figure 2.1. The left main coronary artery soon bifurcates into the left anterior descending coronary artery (LAD) and the left circumflex coronary artery (LCX). Blood flow within the coronary circulation is regulated by:

- coronary perfusion pressure,
- intramyocardial pressure,
- and control of smooth muscle [16].

Coronary perfusion pressure is the pressure difference between the aorta, where the coronary arteries originate, and the right atrium into which the coronary circulation empties. Intramyocardial pressure is due to the numerous branches extending from the main coronary arteries into the myocardium. As the heart contracts during systole, there is little blood flow in the coronary circulation as the vessels are compressed. Consequently, the coronary circulation receives most of its perfusion

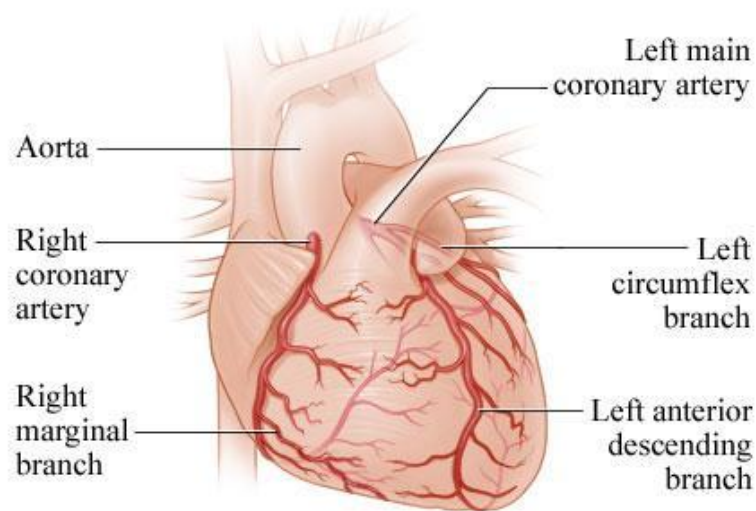


Figure 2.1: Location of the main coronary arteries of the heart [18].

during diastole as the heart muscle relaxes and high pressure blood flows into the coronary arteries due to aortic recoil [16,17]. Smooth muscle tone is controlled by the autonomic nervous system and metabolites [16].

2.1.1 Structure and Function

The structure and function of arteries is highly dependent on location within the arterial tree. Arteries are generally divided into two categories; elastic arteries and muscular arteries. Elastic arteries are the largest arteries in the body and include the aorta, common carotid, and common iliac arteries. Muscular arteries are generally small- and medium-sized blood vessels and include the coronary and brachial arteries. Like all arteries, the small-diameter muscular coronary arteries consist of three layers; the tunica intima, tunica media, and tunica adventitia as shown in Figure 2.2.

The tunica intima lines the lumen of the vessel and consists of a basal lamina and a monolayer of endothelial cells. It forms a smooth non-thrombogenic lining and acts as a selective barrier to plasma lipids and lipoproteins. The thin basal lamina, comprising of type IV collagen, fibronectin, proteoglycans, and laminin, provides a surface on which endothelial cells grow and a limited amount of structural support. Endothelial cells are flat, elongated cells, 10 - 15 μm wide and 25 - 50 μm long, orientated in the direction of blood flow [16]. Additionally, in coronary arteries there

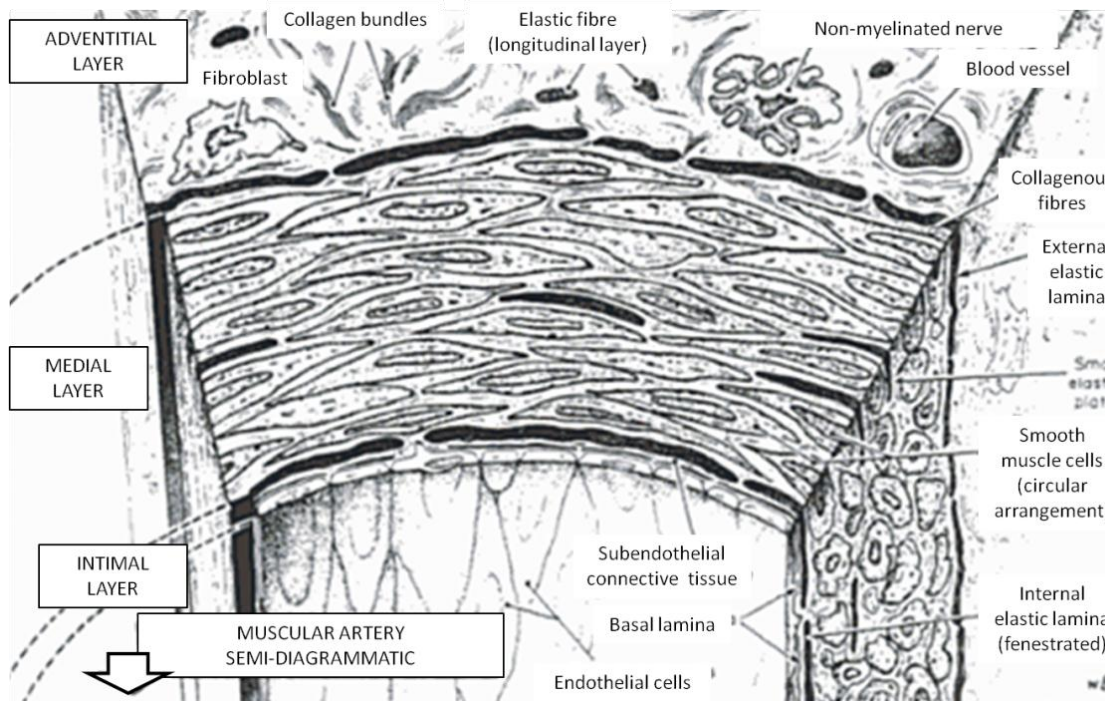


Figure 2.2: Schematic of the components the muscular arterial wall (adapted from [19]).

is a subendothelial layer of connective tissue and longitudinally orientated smooth muscle cells [16,19].

The internal elastic lamina, consisting of a fenestrated sheet of elastin, separates the tunica intima from the tunica media. A major differentiation between muscular and elastic arteries is the presence or absence of elastic laminae, sheets of elastin fibres, in the tunica media. Depending on the size of the elastic artery, there can be up to sixty layers of concentrically arranged elastic laminae separated by vascular smooth muscle cells (vSMCs). The tunica media of muscular arteries, however, do not contain elastic laminae. Instead, vSMCs, 100 μm long and 5 μm diameter, are the dominant components surrounded by a matrix of loose elastin and collagen fibres [16]. The helical orientation of vSMCs in the media also varies depending on artery type and location within the vasculature. In coronary arteries, vSMC in proximity to the internal elastic laminae are aligned in the longitudinal direction, see Figure 2.3 (A), the orientation of which does not change with vessel dilation or contraction. vSMC orientation becomes increasingly circumferential towards the external elastic lamina, see Figure 2.3 (B), the pitch of which alters upon vessel

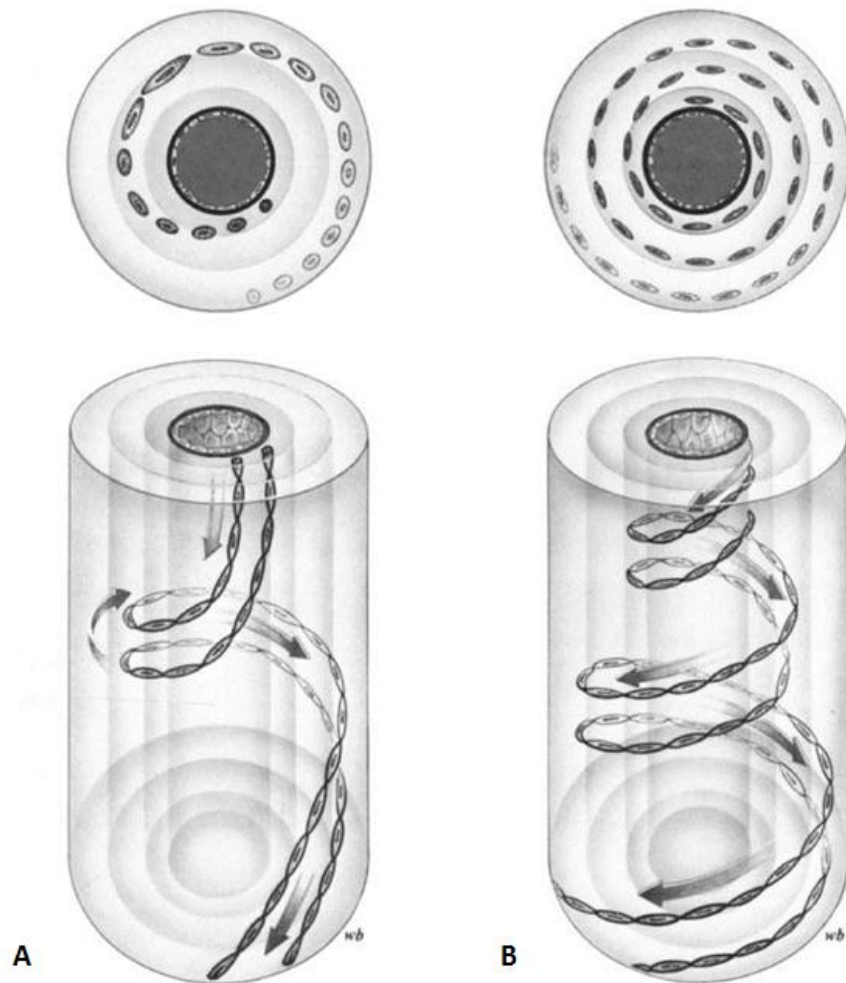


Figure 2.3: Orientation of smooth muscle cells in the media of (A) typical and (B) atypical muscular arteries. Smooth muscle cells of the coronary arteries are arranged in a longitudinal direction close to the internal elastic lamina, similar to (A), moving to a more circumferential orientation towards the external elastic lamina, similar to (B) [19].

contraction or dilation. vSMCs regulate the lumen diameter and synthesise the collagen and elastin fibres of the media [16,19].

The external elastic lamina separates the tunica media from the tunica adventitia. This layer is less distinct in muscular arteries than in elastic arteries. The tunica adventitia anchors the blood vessel to the surrounding tissue, provides stability, and delivers nutrients to the vSMCs in the media. It consists of a dense network of type I collagen bundles and elastin fibres, fibroblasts, nerves, and blood vessels. Fibroblasts maintain the tunica adventitia, in particular the synthesis of type I collagen. The

tunica adventitia of elastic arteries is of the order of 10% of the wall thickness which increases to 50% for muscular arteries [17].

2.1.2 Biomechanical Properties

The complex structure of the arterial wall described above gives rise to a highly non-linear, anisotropic, heterogeneous, viscoelastic material. Arterial biomechanics are dependent on artery type (muscular or elastic) and location within the vasculature. However, general principles apply to all arteries and a range of biomechanical tests are used to determine specific arterial biomechanical properties.

Uniaxial tensile tests are widely used to characterise the non-linear behaviour of arteries [20-23]. Collagen is the main load-bearing element of an artery and provides resistance to systolic blood pressure. Cross-linked elastin polypeptide chains form elastic fibres to provide the elastic recoil of the artery after a pressure pulse. Determined by uniaxial tensile tests, the stress-strain response of arteries exhibit a J-shaped non-linear stress-stiffening behaviour [23], see Figure 2.4 (A). The response of elastin, which undergoes large extensions at low loads, dominates the initial linear region of the curve. Collagen fibres, undulated in their physiological form, straighten out at this early stage [16]. As the collagen fibres are recruited they begin to bear the load and a large increase in stress over small strains is observed. Roach and Burton [24] illustrated the individual contributions of collagen and elastin in human iliac arteries by selective digestion of each protein, see Figure 2.4 (B). Elastin was digested by trypsin resulting in a collagen-based material that stiffened almost immediately under tension and demonstrated little extension. Digesting collagen from the artery using formic acid, the resulting elastin-based material exhibited large, almost linear extension under tension. Elastin fibres can withstand uniaxial extensions up to 150%, yet return to their original formation on unloading [16], whereas collagen can only extend between 10 - 15% [25]. Elastin has a Young's Modulus of 0.06 MPa which is low compared to axially orientated type I collagen exhibiting a stiffness of 100 MPa [25]. The modulus of elasticity or Young's Modulus is defined as the slope of the elastic region of the stress-strain curve. Due to non-linearity of arterial tissue an incremental Young's Modulus must be obtained to determine the stiffness of the material. The elastin dominated toe region is commonly

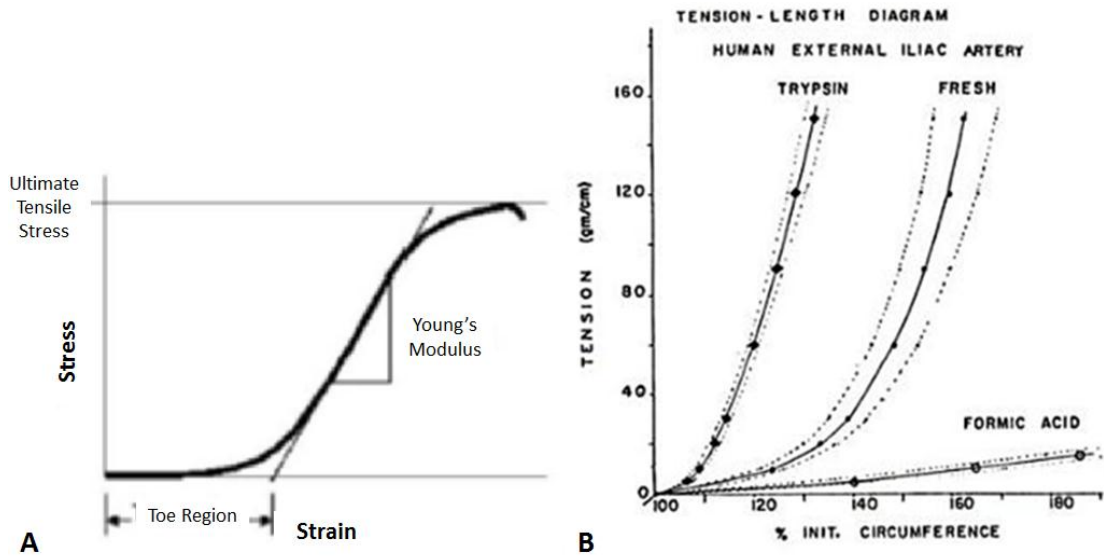


Figure 2.4: Non-linearity of arterial tissue. (A) Typical non-linear stress-strain response of soft tissue to uniaxial tensile test (adapted from [23]). (B) Stress-strain response of fresh arterial tissue and of collagen and elastin as a result of selective digestion [24].

defined by extending the final linear region to the x-axis and recording the intercept [23], see Figure 2.4 (A). In addition to the dominant contributions of collagen and elastin to the stress-strain response, the complex nature of the vascular wall indicates that other ECM elements and the cells, in particular vSMCs, play a role in the response [16]. Van Andel et al. [13] found the difference in the stress-strain response of the main coronary arteries between individuals/pigs was greater than the difference between arteries from the same heart.

Uniaxial tensile tests in one direction do not, however, offer a full characterisation of arterial tissue due to anisotropy. Differing responses are exhibited axially, circumferentially and radially. This is illustrated by the stress-stretch curves of the individual layers of human coronary arteries tested in the longitudinal and circumferential directions [20], see Figure 2.5. The stress-stretch response of each layer is clearly different while individually they exhibit different behaviour in the longitudinal and circumferential directions. Circumferentially orientated intima and adventitia stiffen at a higher stretch than their corresponding longitudinal segments whereas the opposite is true of the media.

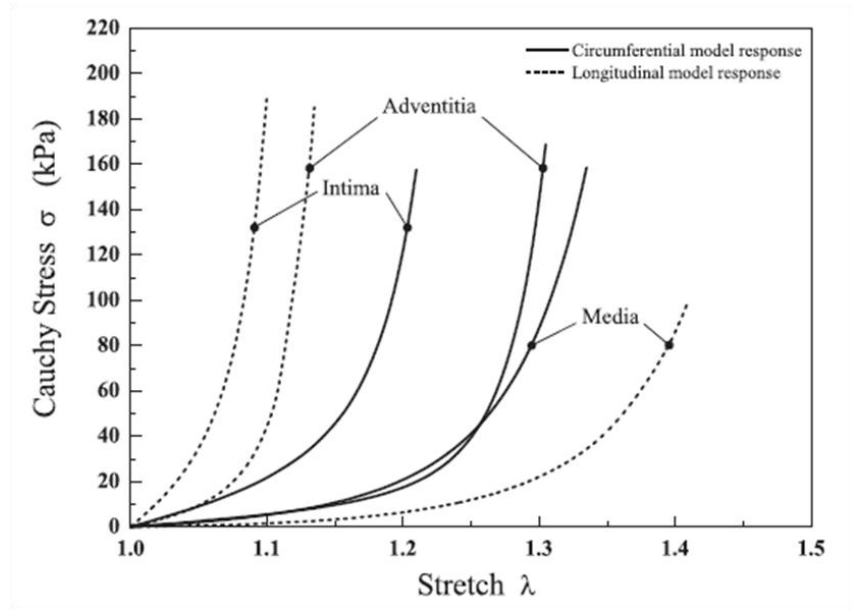


Figure 2.5: Arterial anisotropy: layer-specific longitudinal and circumferential stress-stretch curves for human coronary arteries [20].

An artery is subjected to longitudinal stretch and internal blood pressure in vivo, however, once excised these stresses are released [25,26]. The artery recoils in the longitudinal direction and relaxes circumferentially into its no-load state [25], see Figure 2.6 (A). Bergel et al. [27] identified that the application of longitudinal strain affected the pressure-volume curve, and so, for mechanical tests a pre-stretch should be applied to mimic in situ conditions. The pre-stretch ratio is calculated as the length of the segment in situ divided by the length of the segment in its no-load state. The pre-stretch ratio for arteries is dependent on species and location of the vessel within the arterial tree [28].

Residual stresses remain in the arterial wall when in the no-load configuration. The magnitude of residual stress is dependent on the location of the artery in the arterial tree, vessel size, thickness/radius ratio, curvature of the vessel, and tissue remodelling [25]. The vessel springs open into the zero stress state when cut radially to fully relieve the internal wall stresses [25], see Figure 2.6 (A). A common measure of residual stresses in the artery wall is the opening angle in the zero stress state. This is measured as the angle from the centre point of the inner wall to the outer edges [29,30], see Figure 2.6 (B-C).

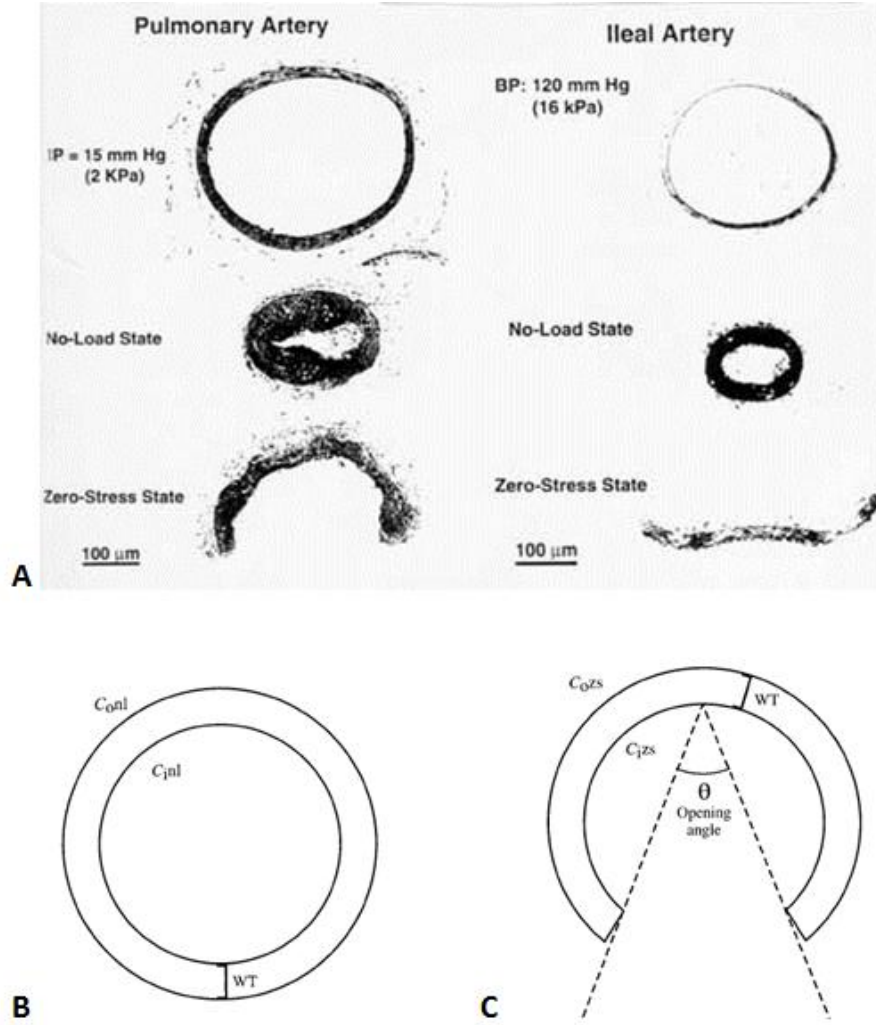


Figure 2.6: Residual stresses in arterial wall. (A) Pulmonary and ileal arteries in their physiological load (top), no-load (middle), and zero stress (bottom) configurations [25]. (B-C) Schematic of the measurement of opening angle; (B) no-load state and (C) zero stress state. WT = wall thickness, C_o = external circumference, C_i = internal circumference, zs = zero stress [30].

Arteries are viscoelastic due to the arterial wall containing viscous fluid-like material and solid material. The properties of viscoelasticity are exhibited by creep, stress relaxation and hysteresis. Creep is the time-dependent change in strain due to a constant load. Stress relaxation is the time-dependent stress response due to a constant strain. Elastin, collagen, and smooth muscle of the arterial wall contribute to stress relaxation [25]. Hysteresis is exhibited by the non-coincidental loading and unloading curves of the stress-strain response and is a result of energy dissipation during the load cycle [26], see Figure 2.7 (A). Additionally, the initial stress-strain

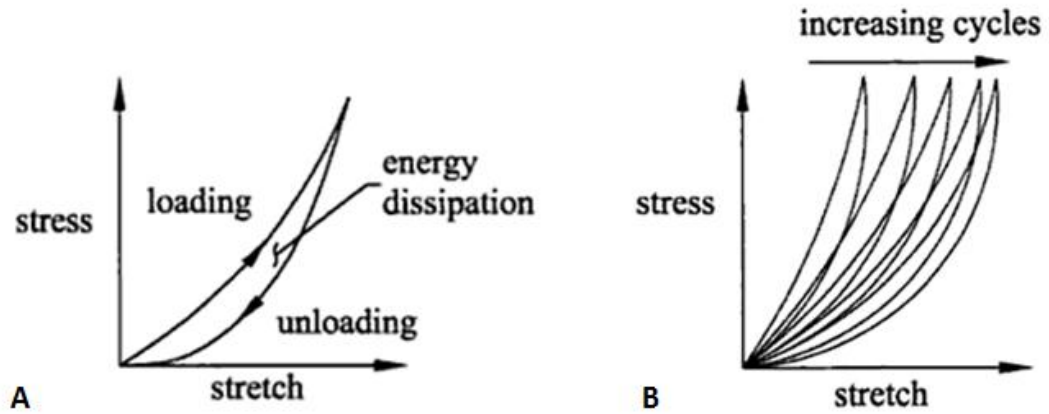


Figure 2.7: (A) Energy dissipation and (B) preconditioning of soft tissue [26].

curve of soft tissue acquired for a specimen is not repeatable upon reloading. Therefore, preconditioning is carried out to obtain a repeatable reference state for stress-strain characteristics [31]. A cyclic test imposed upon arterial tissue exhibits a hysteresis loop, however this loop decreases with following cycles. The hysteresis loops decrease rapidly at first before reaching a steady state. The number of preconditioning cycles required to reach this state vary from as few as 2 to greater than 10 cycles, depending on the type of material being studied and the type of test being performed [26,31], see Figure 2.7 (B).

2.1.3 Porcine Coronary Arteries

The use of human tissue in research is limited due to ethical, health and safety, and availability reasons. Unlimited availability, rapid growth, low infection risk, and low cost have led to the extensive use of pigs in research [32]. The pig is widely used in cardiovascular research due to the perception that the circulatory system is almost identical to that of humans. In fact, the quadrupedal stance of the pig versus the bipedal stance for humans results in gross morphological differences in the hearts of the two species. Despite this, the porcine coronary arterial circulation is almost anatomically identical to the human circulation [33,34]. A combination of these factors led to the use of porcine hearts and, in particular, porcine coronary arteries in this study.

A number of biomechanical differences between human and porcine coronary arteries have been identified in a limited number of studies in the literature, see Table 2.1. Porcine arteries in general have a higher pre-stretch ratio than human arteries [13,20]. The pre-stretch ratio of human coronary arteries has been reported between 1.04 [20] and 1.1 [13], while porcine coronary arteries have a pre-stretch ratio of 1.3 [13]. Roy et al. [36] showed that altering the pre-stretch of a vessel can influence the vessel compliance. Therefore, a blood vessel or TEBV could potentially be tuned to match the compliance of the native vessel. Additionally, van Andel et al. [13] found porcine coronary arteries to be 3 times more elastic than human coronary arteries. However, in general, arteries of young pigs, usually 6 to 9 months, and aged human arteries are tested. With aging, human arteries exhibit intimal and medial thickening and an increase in the collagen:elastin ratio resulting in a stiffer vessel [16]. For both human and porcine coronary arteries, the variability in the stress-strain response of arteries from the same heart (LAD, RCA, and LCX) has been found to be lower than the variability between individuals [13].

Table 2.1: Comparison of human (HCA) and porcine (PCA) coronary artery properties. n/a: not available.

	Units	HCA	PCA
Wall Thickness	mm	0.87 ± 0.23 [20] 0.89 ± 0.21 [13]	0.74 ± 0.17 [13] 0.22 ± 0.03 [30]
External Diameter	mm	4.5 ± 0.3 [20] 3.54 ± 0.51 [13]	3.44 ± 0.39 [13] 2.43 ± 0.34 [30]
Pre-stretch	-	1.04 [20] 1.1 [13]	1.3 [13]
Burst Pressure	mmHg	Up to 200 mmHg* [13]	Up to 300 mmHg* [13]
Elasticity	%	20 [13]	60 – 70 [13]
Opening Angle	°	n/a	75 [35] 169 [30]

*Established during inflation test. Tears in tissue occurred above the stated pressures.

2.1.4 Coronary Artery Disease and Interventions

As previously stated, coronary artery disease (CAD) is the most prevalent form of cardiovascular disease and the leading cause of death in the western world. Atherosclerosis is a type of arteriosclerosis which affects medium- and large-sized arteries. It is a chronic inflammatory disease resulting in a localised thickening, stiffening, and remodelling of the intima of arteries. The development and progression of atherosclerosis is highly dependent on the location of the artery in the vasculature where local structural components and mechanical forces act [37]. In particular, arteries with complex geometries such as coronary, cerebral, and carotid arteries are susceptible to atherosclerosis due to blood flow hemodynamics and mechanical forces at localised sites. The curvature and many bifurcations of the coronary arteries can result in adaptive intimal thickening in localised areas, however, this is not an indication of disease [38], see Figure 2.8. Although regions of intimal thickening are more susceptible to the development of atherosclerosis than other regions, the progression of the disease is not dependent on it.

The abnormal expression of adhesion molecules due to a dysfunction of the endothelium results in a localised accumulation of monocytes from the blood stream. Upon migration into the intima, the monocytes convert to macrophages and then foam cells following the uptake of low-density lipoproteins. Foam cells secrete growth factors and chemotactic factors resulting in the migration of proliferating smooth muscle cells from the media and an increase in the uptake of macrophages and T-cells from the circulating blood [39,40]. Initially, the artery responds to the thickening of the inner wall by vasodilation to maintain the patency of the vessel lumen. The lesion will eventually intrude on the vessel lumen with a continuation of the inflammatory process, increasing the size of the lesion [40].

In a series of reports the American Heart Association classified atherosclerotic lesions based on histological composition ranging in increasing severity from types I to VIII [37,41], see Figure 2.8. Types I to III, the early stages of the disease, are clinically silent and do not impede the lumen of the vessel. Type IV atheroma and type V fibroatheroma differ in the nature of the fibromuscular layer facing the lumen of the vessel. In type IV lesions this layer is composed mainly of the original intima,

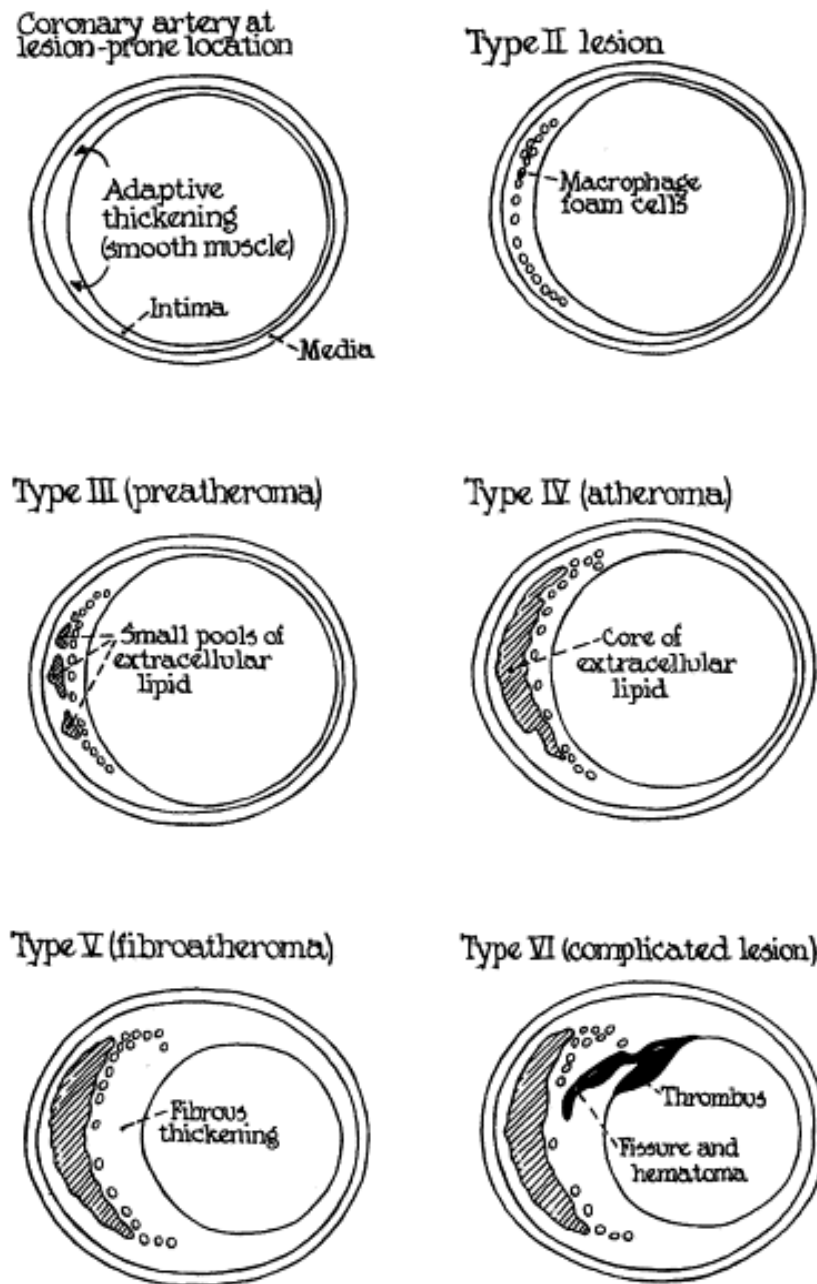


Figure 2.8: Schematic of intimal hyperplasia and stages of atherosclerosis. Six identical cross-sections of left anterior descending coronary arteries indicating intimal thickening and atherosclerosis at this location [41].

although it may have undergone some intimal thickening depending on location within the arterial tree. However, in type V lesions, a thick fibrous cap is formed consisting of smooth muscle cells and connective tissue, in particular collagen. Type IV and V lesions can develop fissures, hematoma, and/or thrombus, progressing the

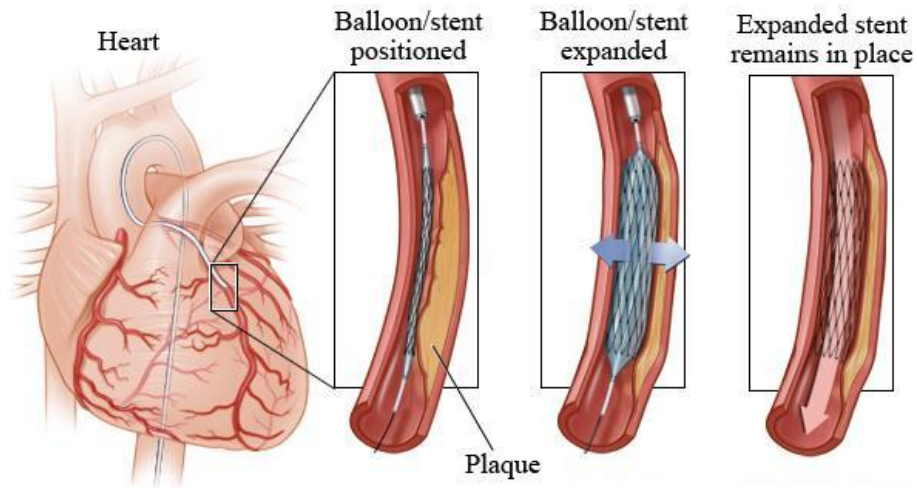


Figure 2.9: Percutaneous transluminal coronary angioplasty with stenting of the left anterior descending coronary artery (adapted from [42]).

disease to type VI with increased lesion size and decreased lumen patency. The lipid core has calcified in type VII lesions and in type VIII there is no lipid core and fibrous tissue dominates.

The onset of CAD ranges from symptomless to severe depending on the degree of the occlusion. Mild onset of CAD is indicated by angina pectoris, where transient or intermittent ischemia and vessel occlusion occurs due to thrombus formation. Myocardial infarctions are severe, acute events during which blood supply is reduced or cut off to areas of the myocardium. The majority of myocardial infarctions are caused by the fibrous cap breaking open and releasing the contents of the lipid core which leads to blood clot formation [17]. This clot can lead to a total or partial stenosis in a region of the coronary circulation resulting in a localised reduction of blood flow to the myocardium [17].

Many coronary artery stenotic lesions, diagnosed by coronary angiography, can be treated using minimally-invasive techniques. Percutaneous transluminal coronary angioplasty (PTCA) is a minimally-invasive procedure where a balloon-tipped catheter is guided to the diseased area. The balloon is inflated, dilating and reopening the narrowed vessel, and the balloon is subsequently deflated and removed [43]. Stenting is a variation of PTCA where a cylindrical scaffold is crimped over the balloon. Inflating the balloon expands the stent which remains permanently in the vessel to maintain vessel patency as shown in Figure 2.9. Angioplasty is associated

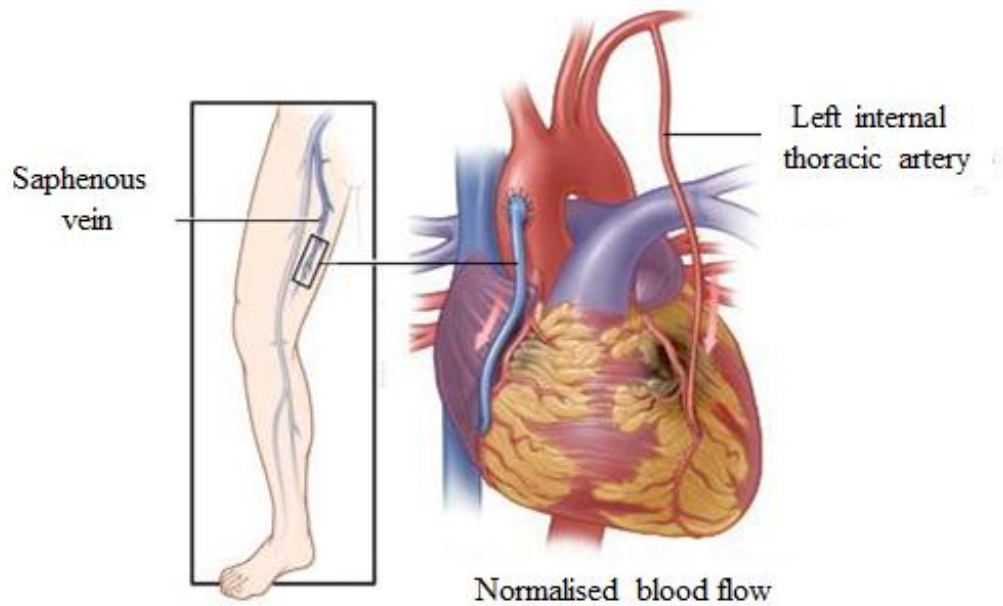


Figure 2.10: Coronary artery bypass grafting of stenosed coronary arteries using autologous grafts; the saphenous vein for the right coronary artery and the left internal thoracic artery for the left anterior descending coronary artery [42].

with low risk, short recovery time, and minimal hospitalisation; however, it is not always indicated as the optimum approach for revascularisation and has limited long-term success [44].

Coronary artery bypass grafting (CABG) is the optimum treatment where there is stenosis of the left main coronary artery or multiple stenoses [45-47]. CABG is an invasive surgical procedure requiring open heart surgery and is therefore a higher risk procedure requiring longer hospitalisation and recovery time. However, the American Heart Association reported a mortality rate of less than 1% in patients less than 65 years old with no severe left ventricular dysfunction or congestive heart failure. Additionally, 5- and 10-year survival rates were reported to be 92% and 81%, respectively [47]. Oxygen-rich blood is rerouted from the aorta around the stenosis through a graft to the coronary artery, see Figure 2.10. Due to the nature of the vessel being replaced, for small-diameter (< 6 mm) applications such as CABG autologous grafts are the preferred option. Segments of the greater saphenous vein and the in situ left internal thoracic artery have the highest long-term success rates as autologous blood vessel grafts to-date [45]. 40 - 60% of vein grafts and 90% of left

internal thoracic arteries are patent within 10 years of bypass surgery [47]. Other natural grafts used include bovine left internal thoracic arteries (50% patency at 1 year) and cryopreserved homologous saphenous vein and glutaraldehyde-treated homologous umbilical vein (50% at 3 to 13 months) [47]. In certain circumstances, where autologous grafts are unavailable or unsuitable due to previous myocardial revascularisation, peripheral arterial reconstruction, or varicose vein ligation procedures, synthetic polymer grafts are required [45]. Polyethylene terephthalate (PET or Dacron) and polytetrafluoroethylene (PTFE) grafts are considered as a last resort having limited success. Synthetic grafts are rigid and cannot adapt diameter in response to changes in blood flow as native vessels can. Therefore, the patency of PTFE grafts 1 year post bypass has been reported as 60% [47].

The long-term success rates of CABG procedures are limited due to atherosclerosis and occlusion of the bypass vessel. The failure of these grafts is influenced by a mismatch in the mechanical properties between the bypass grafts and the native vessel [7]. In particular, an incompatibility in compliance, the dimensional change with respect to luminal pressure change, has been identified as a marker for graft failure. A compliance mismatch can lead to intimal hyperplasia at the anastomosis and thrombotic occlusions. Excessive mechanical stress at the anastomosis can result in endothelial wall injury, a precursor for the development of atherosclerosis. In addition, hemodynamic wall shear stress affects the biochemistry and permeability of endothelial cells. A low mean wall shear stress has been identified as a marker for atherosclerotic development and intimal hyperplasia.

The high incidence of CAD and the limited long-term outcome of the intervention procedures points to the need for an improved bypass graft with matching biomechanical and structural properties.

2.2 Tissue Engineering and Tissue Engineered Blood Vessels

Organ or tissue transplants, surgical reconstruction, and support via a mechanical device are the most common treatments for tissues or organs impaired as a result of

disease, trauma, or congenital defects [48]. While these treatments assist in maintaining and sustaining life, they are not ideal solutions. Each year an increasing number of people die while on organ transplant waiting lists [14,49,50]. In Ireland, 20 - 35% of patients on the heart transplant list die while waiting, whereas of the small proportion of suitable donors only 24 - 42% donate [50]. These statistics are indicative of all types of transplant procedures worldwide demonstrating that the critical shortage of donors severely limits transplant procedures. Surgical reconstruction is also hampered by a shortage of donor tissue and donor site morbidity [51]. Mechanical devices, such as kidney dialysis machines, cannot match the functionality of the organs they replace. Only a limited range of the functions required to maintain an organs' viability can be performed leading to long-term deterioration [48]. These limitations have stimulated research into the development of alternative solutions.

Tissue engineering is a multidisciplinary field combining the specialities of mechanical, material, and chemical engineering with molecular and cellular biology to develop biological substitutes which restore, maintain, or improve tissue or organ function [48,52,53]. The objective is the design, construction, modification, growth, and maintenance of living tissues to replace or augment diseased, injured, or defective tissue. Progress is being made in the development of tissue engineered organs and tissues for the human body including blood vessels [36,54,55], heart valves [56,57], ligaments and tendons [58], cartilage [59], bone [60], bladder [61], skin [62], and trachea [63].

The most common tissue engineering approach involves seeding cells onto a scaffold or matrix which is cultivated in vitro, see Figure 2.11. Autologous cells, isolated from the patient, are the ideal cell source as they do not elicit an immune response and hence avoid potential rejection. The scaffold, either natural or synthetic, provides structural and mechanical stability, determines the 3-D morphology, supports tissue ingrowth, and aids in the formation of tissue structure. External stimuli including mechanical stimulation and chemical factors enhance tissue development.

There is much research into the development of a small-diameter tissue engineered blood vessel (TEBV) to provide an alternative graft for CABG and other small-diameter applications to overcome current limitations and to improve

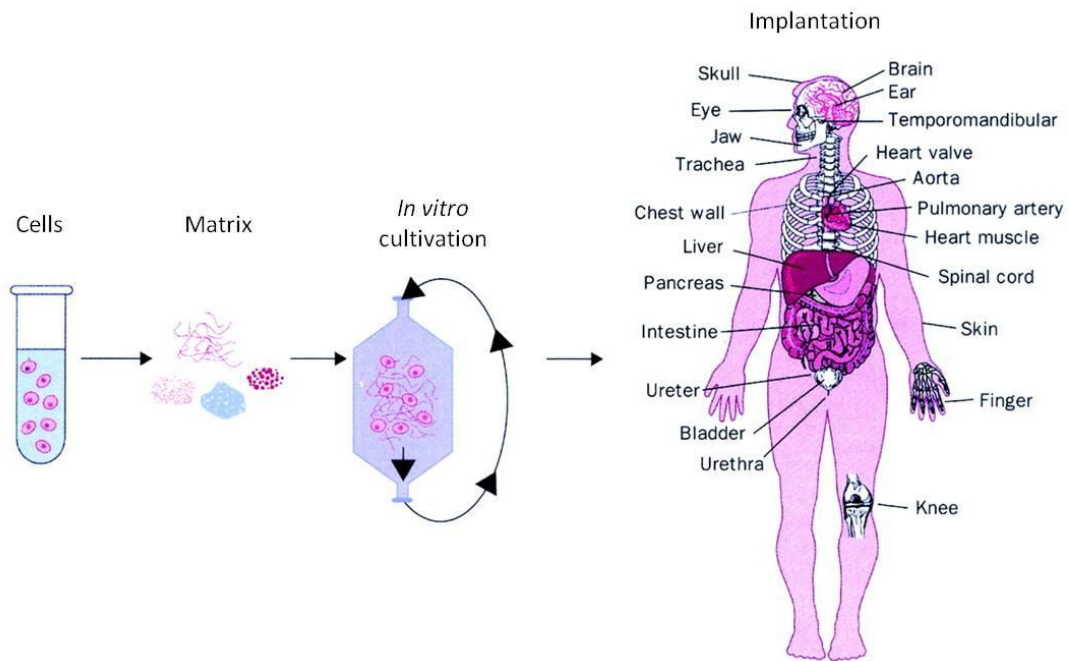


Figure 2.11: In vitro tissue engineering schematic (adapted from [51]).

long-term patency rates [8,36,54,55,64-66]. In 1986, Weinberg and Bell [67] produced the first completely biological TEBV comprising of a collagen gel, endothelial cells, smooth muscle cells and adventitial fibroblasts. Unfortunately, without the support of a Dacron mesh, this graft could support pressures of less than 10 mmHg. Since then, there have been many advances in the field of TEBVs. It is critically important for the development of a small-diameter TEBV that the structural, chemical and mechanical properties are equivalent to the native vessel to maintain patency. Key requirements include haemocompatibility, sufficient mechanical strength, compliance and elasticity, and biocompatibility. These requirements are proving to be a challenge and no truly compatible TEBV has been developed to date.

2.2.1 Tissue Engineering Blood Vessel Scaffolds

Current research into a suitable scaffold material for a viable TEBV is divided into two categories; synthetic polymers and natural matrix proteins. Biological scaffolds have the advantage that they are generally biocompatible, naturally occurring, non-toxic materials and are therefore favourable for cell attachment. However, they are susceptible to rapid degradation, weak mechanical properties, batch

inconsistencies, and the possibility of disease transmission [68]. Synthetic materials for TEBVs are biodegradable or non-biodegradable. The main advantages of non-biological scaffolds include accurate control of material properties, ready availability, cost effectiveness, ease of manufacture, and reduced batch variations. Toxic residuals as a result of the manufacturing process and poor cellular interaction, however, are among the disadvantages [68]. For biodegradable materials there is the added disadvantage of the by-products of scaffold degradation eliciting an inflammatory response [68].

Synthetic materials implanted often lead to an inflammatory response by the host and can trigger a process of events including the activation of platelets and leukocytes and the initiation of thrombogenesis and intimal hyperplasia [14]. These responses are a major cause of failure for implanted synthetic grafts. A solution is to seed endothelial cells on the blood contacting surface of the graft preventing the host blood from contacting the polymer, although there is difficulty surrounding the retention of the cells once implanted [14]. There is a wide range of non-biodegradable synthetic materials for vascular grafts, the most widely accepted being polyethylene terephthalate (PET) or Dacron for medium- to large-size grafts and expanded polytetrafluoroethylene (ePTFE) for small-diameter vascular grafts [7,69,70]. Biodegradable synthetic materials used as vascular grafts or scaffolds include polyglycolic acid (PGA) [71-73] and polylactic acid [74]. One approach for the use of biodegradable polymer materials is the implantation of a graft which degrades over time as tissue remodelling occurs in and around the graft. The second approach is the growth of vascular tissue *ex vivo* on a biodegradable scaffold prior to implantation [69]. However, studies of biodegradable TEBV have reported lower mechanical properties in comparison to native blood vessels [72] and a lack of elastin fibres [73]. Dahl et al. [72] compared porcine common carotid arteries to engineered PGA scaffolds seeded with porcine carotid SMCs. The engineered vessels had statistically significant lower burst pressure (107 ± 14 mmHg vs. 443 ± 55 mmHg), lower maximum stress (1.4 ± 0.25 MPa vs. 6.58 ± 0.97 MPa), and lower mean compliance (3.5 ± 0.2 % per 100 mmHg vs. 18.7 ± 4.1 % per 100 mmHg) than the native vessel.

TEBV scaffolds consisting of natural matrix proteins are achieved by a gelation process or electrospinning of proteins, or the decellularisation of natural allogeneic or xenogeneic tissue. Biological scaffolds are generally composed of only one or some extracellular matrix components, and therefore synthesised scaffolds often do not prove to be an exact biological and structural match for native vessels as they lack other required elements. Nevertheless, they have improved compliance characteristics in comparison to synthetic materials. Appropriate decellularisation, however, can result in the natural matrix scaffold of allogeneic or xenogeneic tissue containing all of the extracellular matrix components. Decellularisation of blood vessels will be discussed further in Section 2.3.

The extracellular matrix (ECM), a complex mixture of functional and structural proteins, remains once cells are removed from an organ or tissue [12]. The ECM is composed mainly of proteins, including collagen, elastin, fibronectin, laminin, and glycosaminoglycans [26]. It is maintained by the cells it encompasses, such as fibroblasts and vSMCs. The functions of the ECM are to maintain the shape of a tissue by providing it with strength and resilience, to provide a biologically active scaffold on which the cells can migrate and adhere, to regulate cell phenotype, to act as an anchor for proteins including growth factors and enzymes, and to allow the diffusion of nutrients, ions, hormones and metabolites between the capillary network and cells through its aqueous environment [26]. The composition and ultrastructure of the ECM is dependent on the tissue from which it is harvested, the species of origin, and decellularisation method. ECM has been derived and utilised in tissue engineering applications from tissues including blood vessels, skin, nerves, skeletal muscle, ligaments, and liver [12].

Collagen is the main protein in the body, consisting of approximately 25 – 30% of all protein [26]. There are many different forms of collagen; types I, II, III, and IV being the most common in the body, while types V, VI, and VIII are also present. The ECM of vascular tissue contains type IV in the tunica intima, types I, III and V in the tunica media and type I in the tunica adventitia. Type IV collagen occurs as a porous network and is found in the basement membrane of all vascular tissues. It provides a scaffold for the attachment of endothelial cells, with the adhesion molecules fibronectin and laminin aiding cell attachment. Type I and type III collagen exist in

the form of fibres and give structural support when in tension. Type V collagen is associated with the vSMCs in the tunica media. In the vasculature, types I and III are synthesised by the smooth muscle cells and fibroblasts while type IV is synthesized by the endothelial cells. The amino acid sequence of type I collagen has remained highly conserved through evolution leading to it being a primary candidate for xenogeneic and allogeneic scaffolds exhibiting low antigenicity [75]. Collagen is important for biological and mechanical characteristics of vascular substitutes, however, it has a negative inflammatory and thrombogenic response. Collagen contact with blood activates leukocytes and platelets, triggering thrombosis and intimal hyperplasia, to a greater degree than basal lamina and elastic laminae [76]. Therefore it should not be used as a blood-contacting surface in a vascular graft. However, collagen may have a positive role in the revascularisation of a matrix given that a collagen matrix attracts more vSMCs than the elastic laminae [76]. Without supplementary support, however, a collagen gel is unable to support physiological pressures [67].

Vascular elastin is synthesized mainly by vSMCs, but also by specialised fibroblasts and possibly endothelial cells [26]. It forms a mesh-like structure that is loose, amorphous, but highly cross-linked resulting in its elastic response over large deformations. It also inhibits the proliferation and migration of vSMCs preventing the formation of a neointima and the stenosis of arteries [68]. The degradation of elastin in arteries plays a primary role in the formation of aneurysms and vascular dissections. Liu et al. [76] investigated the effect of allogeneic elastic laminae upon implantation. Primarily composed of elastin, it plays a role in structural stability and elasticity, and regulation of cellular activity of blood vessels. It was found that elastic laminae had a neutral thrombogenic and inflammatory response and, therefore, is a potential blood-contacting surface for a vascular graft. Elastin has been incorporated into biological scaffolds in combination with collagen through electrospinning [77].

Fibrin gel has also shown potential as an ECM scaffold due to good cell adhesion properties and increased production of ECM proteins [74,78]. It consists of fibrinogen and thrombin which can be isolated from recipient blood. However, similar to collagen, it requires support in order to withstand physiological pressures. Tschöke et al. [74] augmented the fibrin gel with a biodegradable poly(L/D)lactide

mesh. Nevertheless, mechanical properties were still inadequate for the graft to perform as a TEBV. Unseeded grafts achieved a burst pressure of 21 ± 6 mmHg. A maximum burst pressure of 466 ± 78 mmHg was achieved after seeding the graft with a combination of smooth muscle cells, endothelial cells and fibroblasts cultured in a bioreactor for 21 days.

A further biological material which has been examined as a potential TEBV scaffold is bacterial cellulose which can be fabricated into tubes for small-diameter applications [79]. This biomaterial has exhibited favourable biological and mechanical properties including low thrombogenicity, favourable cell attachment capabilities, non-linearity, and good mechanical strength.

2.2.2 Cell Source for TEBV Scaffold

Cells are required within a TEBV scaffold for tissue growth, function, and repair. The cells used should be non-immunogenic, highly proliferative, easy to harvest, and be capable of differentiating into specific cell types [80]. For TEBVs, endothelial cells (ECs) are required as a non-thrombogenic blood-scaffold interface, and smooth muscle cells are required to produce extracellular matrix proteins, in particular collagen and elastin, and to provide smooth muscle tone for the regulation of lumen diameter. Autologous vSMCs and ECs isolated from a blood vessel explant are the ideal candidate for seeding a scaffold. However, they are an inadequate source for in vitro scaffold population due to limited proliferation potential and reduced matrix production of smooth muscle cells, particularly for aged donors [80-82]. The use of xenogeneic and allogeneic vSMCs and ECs is restricted due to the host immune response and potential for disease transmission.

Bone marrow-derived mesenchymal stem cells (MSCs) show great potential as an autologous cell source for the population of TEBV scaffolds. They are easily isolated and rapidly expanded in vitro and have been shown to differentiate into both vSMCs and ECs under certain conditions [82-85]. Additionally, as they do not express the major histocompatibility complex II antigens associated with immune rejection they can potentially be used as an allogeneic cell source. Therefore, MSC-seeded scaffolds may have potential as off-the-shelf TEBVs for emergency procedures.

Another type of adult stem cell, endothelial progenitor cells (EPCs), derived from bone marrow or from umbilical cord blood, have also shown the potential to proliferate, migrate, and differentiate into mature ECs [81,84].

Taking a different approach, L'Heureux et al. [86,87] examined the potential of human fibroblasts as a cell source. Using a sheet-based tissue engineering technique and fibroblasts obtained from human skin they produced a completely biological TEBV without the support of a scaffold. However, the time required to produce these vessels limits their long term potential given that it was approximately 28 weeks.

The advantage of controlled application of mechanical stimuli to encourage tissue maturation and extracellular matrix formation in a cell-seeded scaffold has been established. In a study seeding ovine myofibroblasts and endothelial cells onto a bioabsorbable polymer scaffold for 28 days, DNA and collagen content of scaffolds under pulsatile conditions was significantly greater in comparison to static controls [88]. The pulsatile sections also showed improved mechanical properties over the static control. Burst strength increased continuously to significant levels over static samples (326.3 ± 24 mmHg vs. 50 ± 5 mmHg at 28 days) and improved suture retention (64.3 ± 5 g vs. 12 ± 3 g) and wall thickness (0.73 ± 0.04 mm vs. 0.50 ± 0.1 mm) levels were also observed. While the biomechanical properties obtained in the study presented in [88] were too weak to be suitable for in vivo implantation, the advantages of a pulsatile system were shown. Similar biomechanical trends were found after pulsatile culture conditions of a polyglycolic acid scaffold seeded with smooth muscle cells [89].

2.3 Decellularisation

TEBV scaffolds prepared from the natural arterial scaffold show great potential, provided that the native biomechanical properties are retained. In an effort to produce TEBVs that match the structural, chemical, and biomechanical properties of the native vessel, allogeneic and xenogeneic blood vessels have been decellularised using a range of techniques creating scaffolds for the migration and growth of native endothelial cells and smooth muscle cells [8,10,11,90-92]. Allogeneic and xenogeneic tissues elicit an inflammatory response or immune-mediated rejection;

however the removal of cells can reduce this effect [12]. Many different animals [8,11,29,55,76,85,90,92-94] have been used to determine the viability of decellularisation methods on a variety of blood vessels including the aorta [76,94], carotid artery [8,29,85,90,92], iliac artery [54,94], umbilical artery [95], saphenous vein [55], and iliac vein [94] for the development of a TEBV. However, despite the prevalence of coronary artery disease and the requirement for a suitable small-diameter vascular graft no study has been carried out to-date to examine decellularisation of human or porcine coronary arteries.

Complete decellularisation is accomplished by disrupting the cell membrane, disassociating cellular components from the ECM, solubilising cytoplasmic and nuclear components, and removing cellular fragments and residual chemicals from the remaining ECM [12]. This has been achieved using combinations of chemical, enzymatic, and physical treatments [12]. The minimum decellularisation time reported has been over 4 hours for rat aorta where agitation in sodium hydroxide (NaOH) for 1 hour followed by 3 hours of vigorous agitation in PBS achieved rapid decellularisation [76]. However, the majority of decellularisation protocols take from several days [85,92,94] to weeks [29,90]. Other studies have shown that mechanical properties of biological materials alter dramatically with time once harvested from the body [22,93]. A disadvantage of lengthy decellularisation methods is the timescale over which they occur. The longer the decellularisation time the greater the natural degradation of the tissue which will have an effect on the biomechanical properties of the resulting scaffold. Lengthy decellularisation times may also reduce the clinical and commercial viability of such TEBVs given the high costs associated with prolonged culture periods. Consequently, it is critical to establish a short-term decellularisation method to retain the native biomechanical properties and architecture aiding the development of a potentially viable TEBV.

Physical methods of decellularisation such as snap freezing, mechanical force, and mechanical pressure cause disruption of the cell membrane and the release of cellular components [12]. However, these treatments are generally coupled with a chemical or enzymatic treatment as they are insufficient to accomplish complete cell removal. In fact, freeze-thaw cycles have been employed as an initial step to open up the arterial matrix to allow for better infiltration of the subsequent reagents [85,91].

Physical methods also assist in the rinsing and removal of cellular material from the ECM [12]. Mechanical agitation, using magnetic stirrers, orbital shakers, and low profile rollers, and mechanical sonication are frequently used in conjunction with a chemical treatment to aid with cell lysis and the removal of cellular components [12,55,76,85,91,92,96].

Chemical treatments cause the disruption of cell membranes and the bonds responsible for intercellular and extracellular connections [12]. Alkaline and acid treatments solubilise the cytoplasmic component of cells and disrupt nucleic acids. Deoxycholic acid [97], ammonium hydroxide [29,64,98,99], and sodium hydroxide [76] have been used to decellularise various blood vessels. Non-ionic detergents are widely used due to their inaction on the protein-protein interactions within the tissue architecture while the lipid-lipid and lipid-protein interactions are disrupted [12]. Triton X-100 is the most frequently used non-ionic detergent [29,36,64,65,90, 98-101]. The wide range of concentrations (0.1% [102], 0.25%[100], 1% [29,36,65,90,100], 2% [92]), treatment times (24 hours [65,100], 48 hours [36], 72 hours [29,92,98,99], 96 hours [64], 216 hours [90]), and blood vessels (aorta, carotid artery, and femoral artery) along with other combined methods makes it difficult to assess the effectiveness of Triton X-100. Both Dahl et al. [65] and Fitzpatrick et al. [100] reported ineffective decellularisation after 24 hours of treatment with 1% Triton X-100, although, both treatments were used in conjunction with other methods. Additionally, Zhao et al. [85] reported difficulty with complete cell removal and poor repopulated cell growth with Triton X-100. Ionic detergents solubilise cytoplasmic and nuclear cellular membranes; however, proteins are denatured due to the disruption of protein-protein interactions [12]. The most widely used ionic detergents in decellularisation protocols are sodium dodecyl sulphate [36,54,55,91,100,103] and sodium deoxycholate [54,100,102,104]. SDS achieves almost complete nuclear and cytoplasmic protein removal; however, the tissue structure is disrupted and there is a loss of collagen integrity. Schaner et al. [55] treated human saphenous vein with SDS at concentrations between 0.01% to 0.1% and physical agitation for a period of 16 hours. A concentration of 0.075% SDS or more resulted in the removal of all endothelial cells from the luminal surface of the vein and 94% of cells throughout the cell wall were also removed. Light microscopy

indicated that the collagen fibre content in the vessel wall remained stable while the elastin fibre content decreased slightly. However, Tsai et al. [103] decellularised rat aorta for a period of 2 hours using 0.075% SDS and while there was no quantitative results reported on the resulting matrix, hematoxylin and eosin staining of the cross-section identified an extremely porous, loose connective tissue matrix. Sodium deoxycholate causes even greater disruption of the native tissue structure [12]. Zwitterionic detergents combine the decellularisation characteristics of ionic and non-ionic detergents. Proteins are denatured to a greater extent than non-ionic detergents but to a lesser extent than ionic detergents [12]. 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) has been used to decellularise arterial tissue where the morphology of collagen and elastin remains intact [65,95]. Detergents must be completely removed from the tissue prior to implantation as detergent residuals may be cytotoxic and inhibit cell adhesion, migration, and proliferation [105].

Enzymatic treatments disrupt cell membranes and intercellular and extracellular bonds [12]. Trypsin is commonly used as an enzymatic treatment for decellularisation [10,29,90,92,96,99,104,106]. It is generally used in conjunction with a detergent process; however, Walles et al. [106] used it in isolation. Following 48 hours of treatment, they reported complete decellularisation and the retention of a well-preserved matrix. McFetridge et al. [107] decellularised porcine carotid arteries using a combination of a solvent and Trypsin-EDTA. Prolonged treatment time resulted in ECM damage, where, at 72 hours several sections exhibited complete loss of vessel architecture. Other enzymatic treatments utilised for decellularisation of blood vessels include RNase and DNase [10,85].

Effective decellularisation and matrix preservation is highly dependent on vessel type as demonstrated by Goissis et al. [94] who used an identical alkaline decellularisation method on various canine arteries and veins. The decellularisation procedure was carried out for time periods between 54 and 84 hours. Cell removal and collagen and elastin preservation depended on the vessel type (artery or vein) and vessel location (thoracic aorta, abdominal aorta, femoral iliac artery). Almost complete removal of thoracic aorta and abdominal aorta vSMCs occurred after 36 hours; however, vSMCs remained in the femoral iliac artery after 48 hours of

treatment. The arteries exhibited a high degree of matrix preservation after 48 hours treatment, although there was a reduction and fragmentation of both collagen and elastin. In contrast, the veins showed no resemblance to native vessels. This is due to the thick medial layer and multiple elastic laminae of elastic arteries which is absent in veins. This highlights the need to customise a decellularisation method based on the vessel type and is demonstrated by the wide range of treatment times and concentrations outlined above.

The majority of decellularisation protocols are performed in conjunction with mechanical agitation, mainly in the form of mechanical shakers or stirrers. However, efficient decellularisation is highly dependent on vessel thickness and density [108]. Decellularisation by agitation results in a non-uniform distribution of fluids throughout the thickness of the tissue as the fluid-contacting surfaces are subjected to prolonged and higher concentrations of fluid compared to the inner tissue. Decellularisation by perfusion, therefore, offers a more consistent method through uniform convective flow and a transmural pressure difference encouraging cells and cellular debris out of the tissue.

The shortest time for successful decellularisation in the literature is sodium hydroxide (NaOH) treatment which is a form of alkaline decellularisation. Liu et al. [76] degraded cellular and matrix components of flat specimens of rat aortae. The result was an elastic lamina-collagen scaffold with and without the basal lamina depending on the treatment time. A treatment time of 1 hour followed by 3 hours in phosphate buffered saline to remove residual chemicals and vascular debris resulted in a scaffold without the basal lamina and with the majority of cell nuclei and smooth muscle cell α -actin filaments removed. Tsai et al. [103] decellularised murine aorta in a slightly shorter time, however, the resulting scaffold showed major structural disruption under histological examination.

To the best of the authors' knowledge no study has previously investigated or successfully decellularised small-diameter muscular coronary arteries. However, this blood vessel potentially offers the best match as a TEBV graft. The most common blood vessel used in decellularisation and repopulation studies to produce a TEBV is the elastic carotid artery [8,29,85,90,92] which, depending on the species of origin, can have a considerably larger internal diameter than the coronary arteries and may

prove to be an unsuitable replacement for the muscular coronary arteries. Many of the studies highlighted above focused on the removal of cells from the tissue and overlooked the effect on the biomechanical properties which is vital to obtain a compatible vessel. Additionally, there is no study of the biomechanical properties of decellularised porcine coronary arteries, which could critically inform the development of other grafts.

2.3.1 Biomechanical Properties of Decellularised Scaffolds

The wide range of species, blood vessels, timescales, and decellularisation protocols described above indicate the difficulty in optimising an approach to achieve a decellularised scaffold suitable as a TEBV scaffold. While the removal of cellular components is vital to suppress the immune response, the biomechanical properties of the scaffold are equally important. For TEBVs in particular, the scaffold should withstand physiological pressures and match the compliance of the native vessel. Many decellularisation studies determine the effectiveness of the protocol but perform no biomechanical analysis [10,76,98,103].

Biomechanical properties are altered as a result of decellularisation due to the removal of cellular components and potential damage to the ECM components. However the decellularisation methods outlined above do not take into account the natural degradation of the tissue once removed from the body. Soft tissues, including arteries, are obtained from cadavers or animals for experimental and clinical applications and are stored for short periods of time in physiological solutions [13,31] or frozen over longer periods [23,109]. Stemper et al. [22] reported that following refrigeration of arterial tissue in physiological solution for 48 hours there is significant decrease in the stress at failure and in the Young's Modulus. Chow and Zhang [93] also reported a decrease in the initial slope and an increase in the final slope of the stress-strain response after 48 hours at 4 °C. This demonstrates the need to perform decellularisation over a short time period soon after slaughter as changes occur naturally in the tissue.

Venkatasubramanian et al. [23] performed a study of the preservation methods of arteries. The stress-strain response of segments frozen to -20 °C revealed a shift to

the left of the curve compared to fresh segments. Histological examination highlighted no significant damage to the ECM. However, cell injury was observed in the form of cytoplasmic contraction, nuclear condensation, and 10% cell necrosis. Conversely, Chow and Zhang [93] reported a significant reduction in collagen content after 1 week storage at -20 °C and -80 °C, corresponding to a more distensible tissue with an increased final stiffness. A number of studies have frozen tissue prior to or post decellularisation [8,92,94,99,105,107]. In some cases this was short-term freeze-thaw cycles to initiate decellularisation. However, in others it was the standard method to store tissue for unspecified periods of time yet it was not considered as potentially damaging to the resulting matrix.

A key factor for vascular substitutes is ultimate strength which is commonly measured as the burst pressure; the value at which the vessel ruptures or bursts when pressurised [110]. An inadequate burst pressure could lead to serious implications such as acute rupture and anastomotic degeneration [55]. In an extensive study of age- and risk-appropriate human saphenous veins and internal mammary arteries, which are favourable substitutes for CABG procedures, burst pressures of $1,599 \pm 877$ mmHg and $3,196 \pm 164$ mmHg, respectively, were reported [66]. These results signify the higher ultimate strength of arteries in comparison to veins suggesting that veins are not ideal replacements for arteries. Other studies of the burst pressure of human saphenous veins yield higher results of 2,250 mmHg [70], $1,680 \pm 307$ mmHg [86], and $2,480 \pm 470$ mmHg [55] most likely as a result of younger, healthier vessels. Human umbilical arteries, an option as a TEBV scaffold, were reported to have a burst pressure of approximately 800 mmHg [111], significantly lower than that of saphenous vein and internal mammary artery. All recorded values, however, are multiples of the normal physiological blood pressure range of 80 – 120 mmHg. Decellularised human saphenous vein achieved burst pressures of $2,380 \pm 620$ mmHg, indicating no significant difference to that of non-decellularised samples [55]. Roy et al. [36] found fresh porcine common carotid arteries CCA to have an average burst pressure of 3,124 mmHg while decellularised vessels ruptured at a significantly lower 2,338 mmHg. These results indicate that burst pressures appropriate for implantation are achievable for decellularised materials. Conversely, decellularised porcine carotid arteries undergoing inflation

tests by Heine et al. [104] failed between 120 and 200 mmHg further highlighting the impact of different decellularisation methods on arteries.

The effect of decellularisation on the stress-strain response is dependent on the method of decellularisation and also the origin of the blood vessel. Some studies report a significantly stiffer and less extensible response as a result of decellularisation [29,95,107]. However Fitzpatrick et al. [100] found the response of porcine aorta decellularised by three separate methods to be more distensible with a lower stiffness in comparison to fresh vessels. Williams et al. [29] performed uniaxial tensile tests on fresh and enzymatically decellularised rabbit carotid arteries. While scanning electron microscopy (SEM), transmission electron microscopy and histology showed the preservation of the major components of the ECM, uniaxial tensile tests demonstrated a significant stiffening of the decellularised artery in comparison to fresh vessels. The tensile modulus increased from 2.29 ± 0.24 MPa to 3.48 ± 0.72 MPa following decellularisation. The decellularised tissue also exhibited almost no extension at low loads with the extensibility of native vessels (79.18 ± 5.7 %) decreasing following decellularisation (8.25 ± 0.95 %). Loosening and uncrimping of collagen fibres observed in the adventitia by SEM may have contributed in part to this stiffening.

Quantification of the opening angle before and after decellularisation will provide an insight into the loss or retention of residual stresses within the arterial wall. It has been reported that the opening angle significantly decreases as a result of the removal of smooth muscle cells by decellularisation from the arterial wall [29,36], see Figure 2.12. Williams et al. [29] reported the opening angle of fresh rabbit carotid arteries decrease from 107° to 66.7° following decellularisation while Roy et al. [36] reported a decrease from 69° to 26° in the opening angle of porcine common carotid arteries.

A further parameter of interest is the permeability of the scaffold which must be balanced to allow for the ingrowth of cells into the scaffold and for the transport of nutrients into and waste products out of the scaffold. Additionally, the permeability should not affect the scaffolds ability to withstand physiological blood flow. To the author's knowledge there is only one study in which a measure of permeability has been taken for blood vessels [108]. This was carried out over 72 hours during which

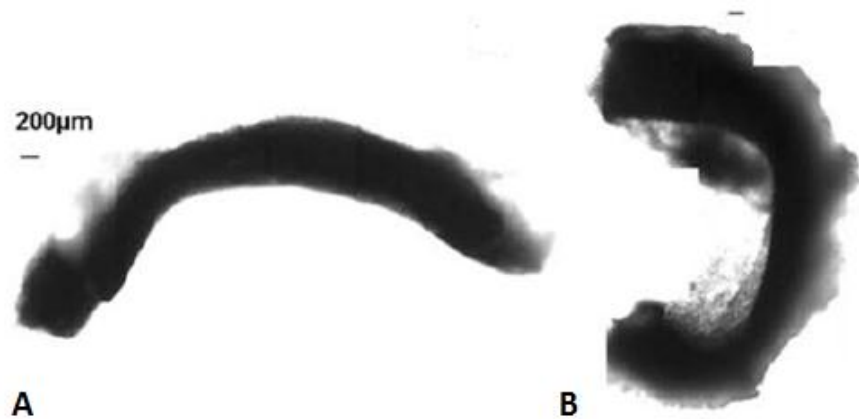


Figure 2.12: Opening angle of (A) non-decellularised and (B) decellularised rabbit carotid artery (adapted from [29]).

the human umbilical vein was decellularised by perfusion and the transmural fluid flow was measured. Therefore, the permeability of the non-decellularised and decellularised scaffold was not directly measured.

Arterial compliance is a measure of vessel distensibility. It is dependent on location within the arterial tree, age, and pathology. For example, a vessel with atherosclerosis will be less compliant, that is, stiffer; while a vessel with an aneurysm may be more compliant [112]. It is critical for the compliance of an implanted graft or a cell-seeded scaffold to match that of the vessel it is being grafted to. A compliance mismatch can result in complications brought about by the discontinuity of blood-flow velocity at the site of the anastomosis. An increase in wall shear stress can result in endothelial cell damage. A decrease in wall shear stress results in areas of blood stagnation leading to increased interaction of the vessel wall and platelets [113]. Due to high local pulsatile stress at the anastomosis, the less compliant the graft/scaffold relative to the blood vessel the greater the occurrence of arterial hypertrophy. The compliance mismatch of bypass grafts in comparison to the artery being replaced is illustrated in Figure 2.13. It is evident that the compliance of the saphenous vein does not match that of the external iliac artery; the vein is a stiffer vessel [9,113]. The synthetic grafts, Dacron and ePTFE, are significantly different to both the artery and the vein. As the saphenous vein is the most successful bypass graft to date, this indicates that there is a correlation between graft compliance and success rate. The graft patency rates for current bypass grafts, both synthetic and

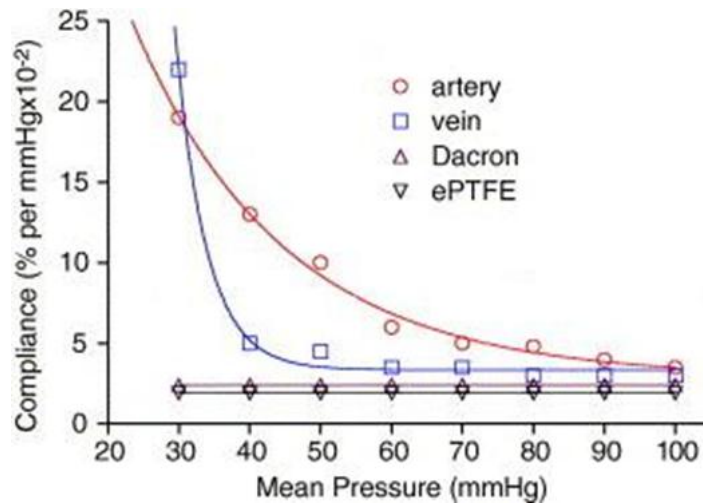


Figure 2.13: Compliance of the internal iliac artery in comparison to the greater saphenous vein, and synthetic materials Dacron and ePTFE [113].

biologic, are identified in Table 2.2 where the relationship between graft compliance and patency is clearly highlighted [113].

Studies where biomechanical properties are assessed compare decellularised matrices to the non-decellularised tissue they were derived from to determine the effect of decellularisation and evaluate the potential of the decellularised scaffold for use as a TEBV scaffold. However, they do not compare the biomechanical properties to the

Table 2.2: Comparison of graft compliance and graft patency for infrainguinal bypass 1 and 2 years postoperative [113].

	Compliance (%mmHg ⁻¹ x10 ⁻²)	1-year patency (%)	2-year patency (%)
Host Artery	5.9	-	-
Saphenous Vein	4.4	88	84
Umbilical Vein	3.7	83	80
Bovine Heterograft	2.6	65	59
Dacron	1.9	65	42
ePTFE	1.6	60	42

vessels they are aiming to replace, in particular the coronary arteries. As previously stated, no study has decellularised coronary arteries and therefore there is no published research on the biomechanical properties of the natural matrix scaffold of coronary arteries.

2.3.2 Repopulation of Decellularised Scaffolds and Resulting Biomechanical Properties

Decellularisation of allogeneic or xenogeneic blood vessels removes the vascular cells and therefore should not elicit a host immune response upon implantation. Consequently, decellularised scaffolds can potentially be implanted as bare grafts, given adequate ultimate strength, where autologous circulating cells and migrating cells from adjacent tissue repopulate the scaffold. Alternatively, the decellularised scaffold can be pre-seeded with suitable cells prior to implantation. The nature of the blood-contacting surface of the scaffold will determine the requirement for endothelial cell-seeding prior to implantation. As previously stated, it is vital that an appropriate non-thrombogenic blood-graft interface be achieved [76]. Many studies have seeded endothelial cells to the luminal surface of decellularised scaffolds in vitro to assess the scaffold capability for cell attachment and monolayer formation [10,64,90,95,96,107]. The endothelial cells were sourced from human saphenous vein [10,64], human umbilical vein [90,95] and porcine aorta [107]. Decellularised human umbilical arteries implanted in rats resulted in thrombus occlusion of five out of the eleven grafts within hours of implantation due to the dense collagen matrix of the scaffold [95]. Quint et al. [114] implanted endothelial cell-seeded and non-seeded decellularised collagen-based scaffolds into pigs. Non-seeded vessels were completely occluded after 30 days (thrombus formation due to collagenous wall); however, seeded vessels were 100% patent. Kaushal et al. [98] reported similar results with endothelial-progenitor cell-seeded decellularised scaffolds which remained patent up to 130 days while non-seeded scaffolds developed thrombus within 15 days.

The structure of the decellularised scaffold has an influence on cell infiltration in terms of porosity/permeability and the barriers of the internal elastic lamina and external elastic lamina. Decellularised umbilical arteries implanted in rats as

abdominal aortic interposition grafts showed no infiltration of the original scaffold after 8 weeks possibly due to the dense collagen matrix [95]. However, decellularised ovine pulmonary trunks and porcine carotid arteries exhibited cellularity throughout the matrices after 20 weeks and 9 weeks, respectively [105]. In an effort to improve cell infiltration of the medial layer of decellularised scaffolds a number of studies have altered the native structure [92,99]. Yazdani et al. [99] removed the adventitial layer following decellularisation to provide seeded vSMCs direct access to the media. However, this resulted in significant differences, such as increased distensibility and decreased elastic modulus, in the stress-strain response in comparison to native arteries due to the removal of type I collagen of the adventitia. Additionally, after 2 weeks of bioreactor culture, vSMC cell count was equal for adventitia-intact and adventitia-denuded scaffolds and it appears that cells did not penetrate the external elastic lamina but formed layers of cells and deposited matrix above it. In addition to removing the adventitial layer, Sheridan et al. [92] introduced needles into the media to directly inject cells. However, without further treatment to increase porosity, seeded vSMCs remained localised to the area they were injected into and did not migrate across elastic laminae.

The majority of studies do not assess biomechanical properties of reseeded decellularised scaffolds, although many would argue that the ability to withstand physiological pressures in vivo in an animal model suggests adequate biomechanical properties. However, an understanding of the biomechanical alteration that occurs as a result of recellularisation could direct future development of TEBV scaffolds. Schenke-Layland et al. [115] performed uniaxial tensile tests of repopulated decellularised porcine pulmonary valves. Ovine endothelial cells and myofibroblasts were seeded onto decellularised porcine valves. Decellularised valves show significant reduction in radial and circumferential tensile strength compared to fresh valves, however, after 16 days in dynamic culture the repopulated valves exhibit similar biomechanical properties to fresh values [115]. Limited biomechanical tests have been performed on repopulated tubular segments of decellularised blood vessels. Recently, Heine et al. [104] performed inflation tests and uniaxial tensile tests on native, decellularised, and in vitro vSMC- and EC-repopulated porcine carotid arteries. Decellularised vessels showed decreased radial distensibility (113%

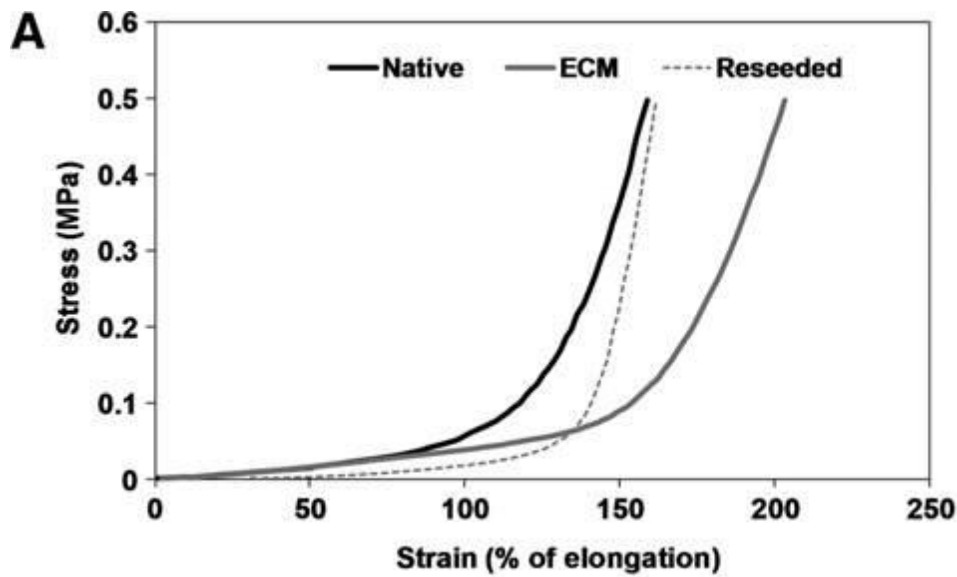


Figure 2.14: *Uniaxial tensile tests of native, decellularised and recellularised porcine carotid arteries* [90].

compared to 135% for native arteries), decreased tensile and tear strength, and increased longitudinal elongation after 2 weeks of culture. However, recellularised arteries displayed improved biomechanical properties with a radial distensibility of 127%, increased tensile strength and tear strength, and comparable longitudinal elongation to native arteries. Dahan et al. [90] demonstrated improved biomechanical properties due to cell seeding of porcine carotid arteries which reversed the effect of decellularisation on the biomechanical properties, see Figure 2.14.

2.4 Summary

The increasing rate and detection of coronary artery disease are furthering the development of biologically and biomechanically equivalent bypass grafts to improve long-term patency. A summary of the native artery, current bypass grafts, and the world-leaders in the development of a small-diameter tissue engineered blood vessel is shown in Table 2.3. The challenges to produce a viable TEBV scaffold/graft for CABG include achieving a small-diameter graft with comparable biomechanical properties, which can withstand cyclic blood pressure, is thrombus resistant, immunologically compatible and readily available for emergency procedures. Widespread advances in the field of tissue engineering, in particular in

the area of decellularisation of blood vessels, are coming to terms with these challenges. In fact, an autologous stem-cell seeded decellularised vein graft was recently implanted in a 9 year old girl as an extrahepatic portal vein bypass which remained patent for 9 months [116]. An additional vein graft was required to restore patency and augment the original graft after 1 year to due to obstruction of the vessel by surrounding tissue. However, while this graft performed admirably as a vein graft with low blood pressures (13 - 20 mmHg) it may not translate to the high pressure coronary arteries.

Decellularisation has been performed on a wide range of blood vessels from different species and using a wide range of protocols. Biomechanical properties of decellularised vessels have been evaluated against the original blood vessel. However, for CABG, it is actually the biomechanical properties of the coronary arteries that should be targeted. Despite the wide range of blood vessels examined as potential scaffolds, there is no study of the natural matrix scaffold of coronary arteries.

A concern with current decellularisation methods is the timescale over which they occur. Along with the potential alteration of biomechanical properties due to decellularisation, the components of the arterial wall naturally degrade over time. Therefore, a short-term decellularisation method is required to maintain the natural biomechanical properties.

Clearly, the development of a small-diameter bypass scaffold/graft prepared from the natural matrix scaffold of coronary arteries may result in a biologically and biomechanically equivalent structure which meets many of the current TEBV challenges.

Table 2.3: Comparison of human coronary arteries to blood vessels, synthetic grafts and tissue engineered blood vessels for use as small-diameter bypass grafts. HCA: human coronary artery, HSV: human saphenous vein, HLITA: human left internal thoracic artery, ePTFE: expanded polytetrafluoroethylene, TEBV: tissue engineered blood vessel, PCA: porcine coronary artery, n/a: not available.

	HCA	HSV	HLITA	Dacron	ePTFE	Niklason TEBV	L'Heureux TEBV
Source	Native vessel	Autograft	Autograft	Synthetic graft	Synthetic graft	Cell-seeded biodegradable PGA scaffold	Sheet-based tissue engineering
Preparation Time	-	Upon explantation	Upon explantation	Off-the-shelf	Off-the-shelf	8.5 weeks [117]	~28 weeks [86]
Length (mm)	20 – 40 [13]	311±101 [119]	180.8±0.07 [118]	unlimited	unlimited	n/a	170 – 210 [66]
Wall Thickness (mm)	0.87±0.23 [20] 0.89±0.21 [13]	0.40±0.12 [119] 0.79±0.02 [120]	0.70±0.07 [13]	n/a	n/a	0.38±0.04 [71]	0.2 – 0.6 [66,87]
Diameter (mm)	4.5±0.3 ¹ [20] 3.54±0.51 ¹ [13]	4.2±0.6 ^{1,2} [119]	3.05±0.5 ¹ [118] 3.28±0.06 ¹ [13]	< 6 ³	< 6 ³	3.1 ³ [117]	1.5 – 6.6 ³ [66,87]
Opening Angle (°)	n/a	~120 ⁴ [120]	n/a	n/a	n/a	n/a	n/a

¹External diameter

³Internal diameter

²Fully distended measurement

⁴Estimated from published graph, values not published

	HCA	HSV	HLITA	Dacron	ePTFE	Niklason TEBV	L'Heureux TEBV
Burst Pressure (mmHg)	Up to 200 ⁵ [13]	1599±877 [66]	3196±1264 [66]	n/a	1323±383 [121]	2150±700 [117]	3399 – 3688 ⁶ [66,87]
Compliance (%mmHg ⁻¹ x10 ⁻²)	n/a	5.0±0.7 [122]	11.5±3.9 [66]	1.8±1.2 [122]	1.2±0.3 [122]	<10 in physiological range [117]	3.4±1.6 [66]
Ultimate Tensile Strength (MPa)	1.30±0.69 ^{6,7} 0.42±0.19 ^{6,8} 0.39±0.14 ^{6,9} [20]	2.2±0.2 [123]	n/a	n/a	14.03±0.72 ⁶	1.2 ¹⁰	1545±526 gram-force ¹¹ [66]
Maximum Incremental Modulus (MPa)	n/a	n/a	n/a	n/a	n/a	66 ¹⁰ [117]	n/a

⁵Established during inflation test.

⁶Longitudinal direction

⁷Adventitia

⁸Media

⁹Intima

¹⁰Diameter-dependent

¹¹Ring segment

Chapter 3 Short-term Decellularisation of a Small-diameter Porcine Coronary Artery

Decellularisation of allogeneic and xenogeneic blood vessels has been widely examined as a method to achieve a structurally and biomechanically compatible scaffold for vascular tissue engineering. This has been driven by the limited long-term patency of synthetic and natural grafts as a result of a biomechanical mismatch with the native vessels they bypass. The use of allogeneic or xenogeneic grafts is limited by the host immune response. However, decellularisation of these blood vessels negates the immunological response whilst potentially maintaining the biomechanical and biological characteristics of the vessel. A wide range of decellularisation methods are employed in the literature, however, the time frames within which these methods are performed are extensive, ranging from days to weeks. As natural degradation of tissue occurs gradually upon removal from the body, achieving decellularisation within a short time period could limit this natural degradation. The most common blood vessels necessitating bypass are the small-diameter muscular coronary arteries. Located in the coronary and interventricular sulci of the heart and with numerous associated branches, these vessels are more difficult to harvest than other blood vessels commonly used as potential tissue engineering blood vessel (TEBV) scaffolds. Consequently, these vessels have not previously been decellularised and assessed as potential TEBV scaffolds. Additionally, many decellularisation methods are performed on lengths and dimensions of blood vessels unsuitable for use as a TEBV. The most common

blood vessel decellularised is the carotid artery which, depending on the source, generally has a larger diameter than human coronary arteries [90,99,107]. Furthermore, the carotid arteries are elastic and, therefore, differ structurally to the muscular coronary arteries. In addition to using a vessel with equivalent dimensions and structure to the coronary arteries, minimising the effect of the decellularisation method on the extracellular matrix is vital to maintain native biomechanical properties and to provide a suitable structure for cell attachment, infiltration and growth. In particular, maintaining an appropriate blood-contacting surface to line the lumen of the vessel is vital to prevent inflammation and thrombus formation upon implantation. This surface should also provide a suitable platform for the attachment and growth of endothelial cells, critical for regulation of a blood vessel.

Therefore, to achieve a structurally and biomechanically equivalent small-diameter natural matrix scaffold suitable for use in coronary bypass surgery a number of criteria should be met:

- Maintain the native tissue structure of the tunica intima, tunica media and tunica adventitia;
- Minimise the time taken to achieve decellularisation;
- Minimise the effect of decellularisation on the extracellular matrix of the tissue, in particular the load-bearing collagen and elastin fibres;
- Maintain a suitable luminal surface.

This chapter presents a method to decellularise lengths of small-diameter, muscular porcine coronary arteries (PCA) using a short-term decellularisation method developed and analysed using histological and immunohistochemical methods.

3.1 Materials and Methods

3.1.1 Tissue Harvesting and Preparation

Hearts from pigs weighing approximately 90 kg and aged 6 months were obtained from an abattoir within one hour of slaughter. The hearts were transported in sterile 0.9% saline (S6546, Sigma-Aldrich) at room temperature and perfused with fresh

0.9% saline to remove residual blood from the atrial and ventricular chambers and the coronary circulation, see Figure 3.1 (A). The small-diameter (internal diameter of < 4 mm) PCA were excised immediately. Segments of the left anterior descending (LAD), left circumflex (LCX) and right (RCA) coronary arteries were exposed using standard dissection tools. Briefly, the tools included Metzenbaum scissors (501253, World Precision Instruments, UK) to spread and separate the epicardium from the PCA, Potts-Smith scissors (503250, World Precision Instruments, UK) for cutting epicardium and branches of PCA, Mosquito forceps (15921, World Precision Instruments, UK) for manipulating excess tissue, and a scalpel to cut the tissue in close proximity to the PCA. A dye was injected into the lumen of the coronary arteries through the openings in the aortic sinuses, see Figure 3.1 (D). The dye highlighted the location of the numerous branches of PCA, each of which was ligated, to allow for fluid to flow through a 3D tubular section, see Figure 3.1 (B-C). Sections of artery up to 60 mm in length were carefully dissected from the epicardium and myocardium and stored in 0.9% saline at room temperature until further use. The coronary arteries excised had similar thicknesses and internal diameters and sections of PCA were only used where the internal diameter was greater than 1.5 mm.

3.1.2 Decellularisation

A chemical decellularisation protocol used by Liu et al. [76] for flat segments of rat aorta was modified for use on PCAs. 0.1M sodium hydroxide (NaOH, S8045, Sigma-Aldrich) was used to disrupt the cells and 0.9% saline removed chemical residue and cellular debris over a period of 3 hours. Preliminary tests of 5 x 5 mm flat sections and short (< 5 mm) cylindrical sections of PCA, with no branches ligated, were carried out by immersion and under constant agitation using a magnetic stirrer to gain an understanding of the effect of 0.1M NaOH, see Figure 3.2.

Segments up to 40 mm in length were required for biomechanical tests and also for potential use as a TEBV scaffold. Therefore, a perfusion system was developed similar to that of Villegas Montoya and McFetridge [108] to improve the infiltration of 0.1M NaOH along the length of the artery, see Figure 3.3. Natural polypropylene barbs (MTLL004-6, Value Plastics, CO, USA), 1.6 mm in diameter, were inserted

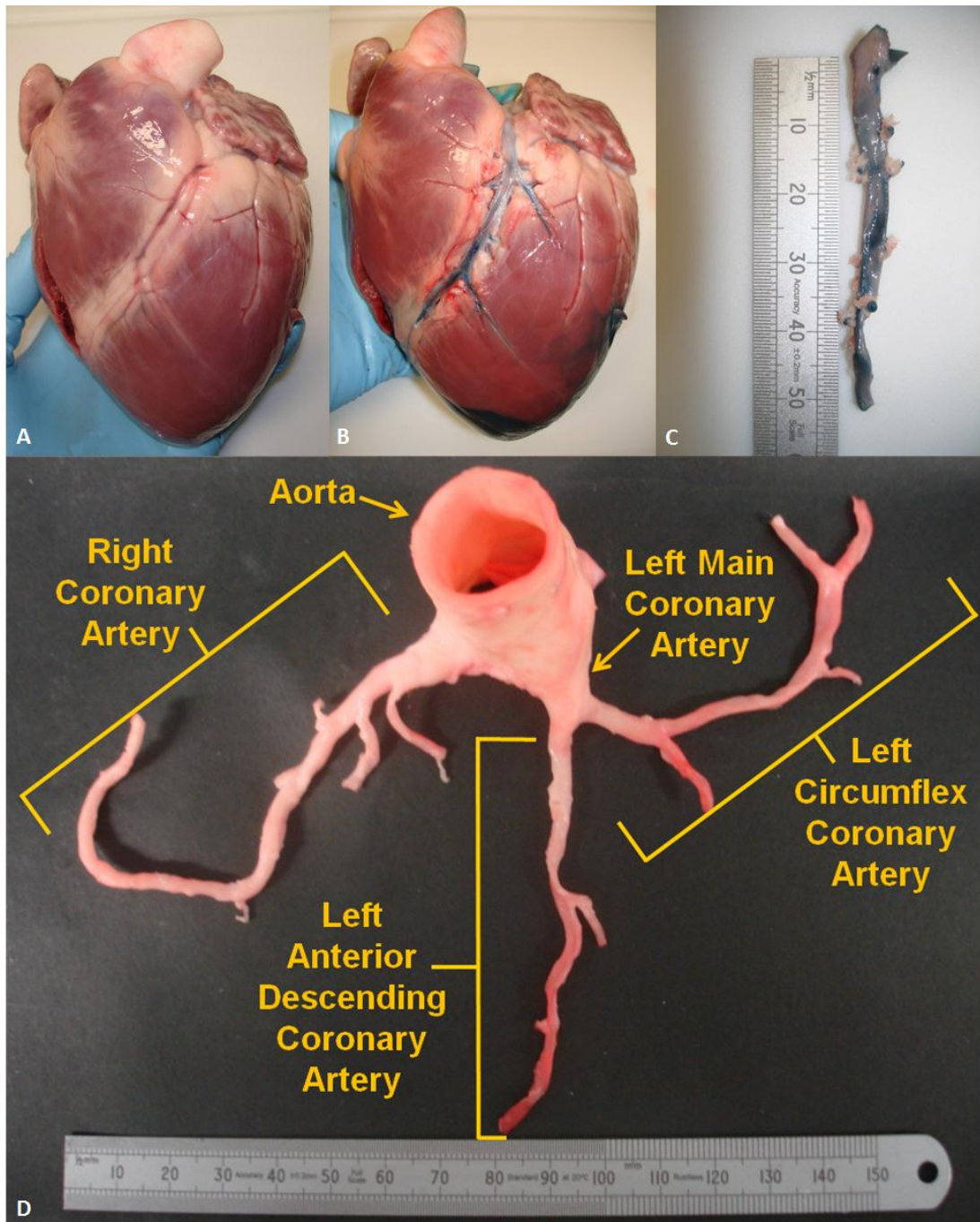


Figure 3.1: Excision of coronary arteries from a porcine heart. (A) An intact heart with epicardium obscuring the coronary arteries; (B) left anterior descending coronary artery in situ with the branches highlighted by injected dye; (C) ex vivo section of left anterior descending coronary artery with all branches ligated; and (D) the main coronary arteries, branching from the aortic sinuses, excised and identified.

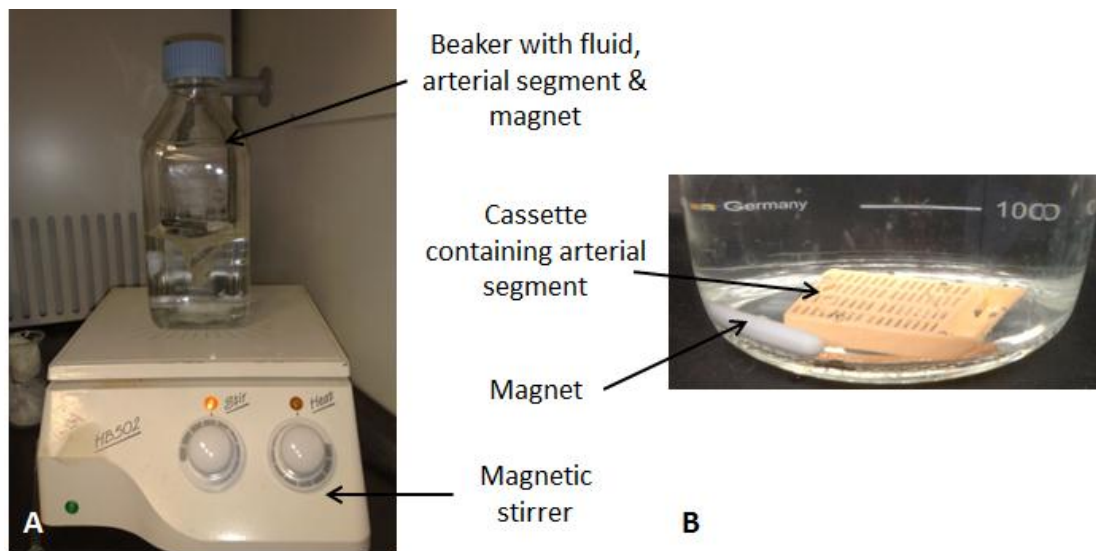


Figure 3.2: Decellularisation by agitation. (A) Flat or cylindrical segments of porcine coronary artery immersed in either 0.1M NaOH or 0.9% saline and agitated by a mechanical stirrer. (B) Close-up of magnet and arterial segment contained in a cassette for protection from the rotating magnet.

into the arterial lumen at either end and mounted horizontally in a flow chamber, see Figure 3.3 (C). A fluid reservoir at a height of 100 mmHg containing either 0.1M NaOH or 0.9% saline was frequently replenished. A peristaltic pump (323, Watson Marlow, UK) circulated the fluid at a frequency of 2 Hz [108] establishing a flow rate of 60 ml/min. A pressure transducer (DTXPlus, BD Medical Systems, NJ, USA) inserted into the lumen of the vessel monitored the pressure. The flow chamber was filled with either 0.1M NaOH or 0.9% saline once the vessel has been monitored for substantial leaks. Four flow chambers could be attached to the perfusion system simultaneously to produce four decellularised PCAs. Sections were treated in 0.1M NaOH for time periods between 1 and 12 hours followed by 3 hours in 0.9% saline to assess for appropriate decellularisation.

3.1.3 Histological Analysis

The effectiveness of the decellularisation procedure was determined by histology. Cross-sections of tissue were stained to visualise the various components of the tissue. Non-decellularised and decellularised sections of PCA were processed for

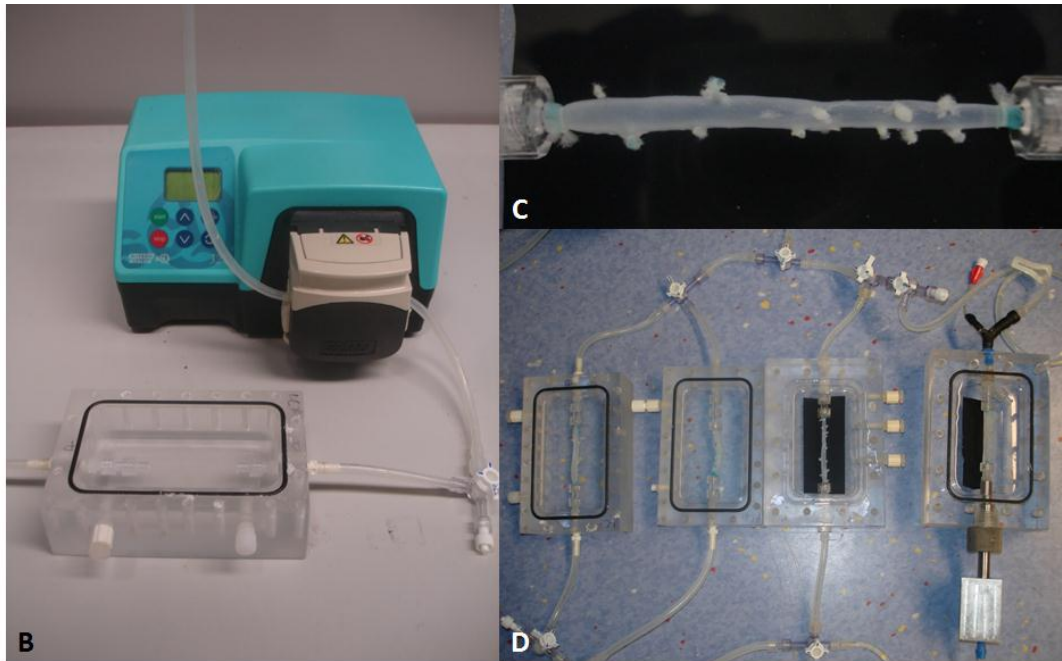
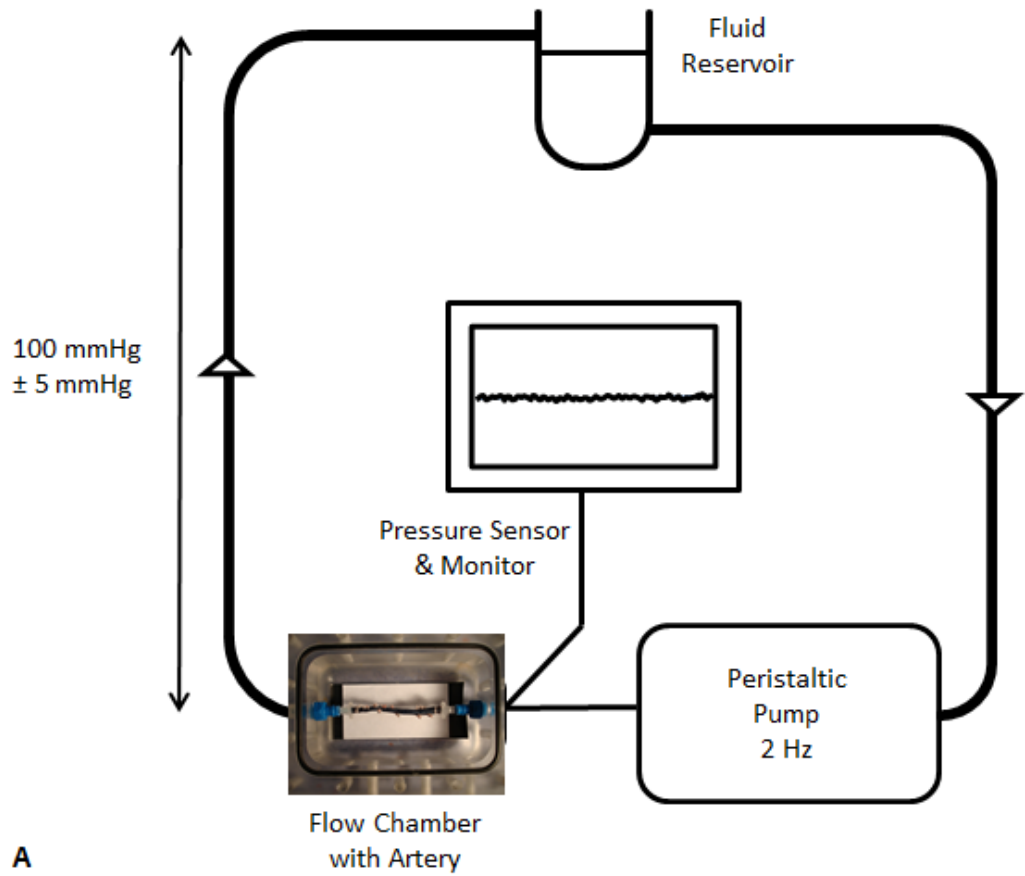


Figure 3.3: Decellularisation by perfusion. (A) Schematic of set-up; (B) peristaltic pump connected to flow chamber; (C) close-up of porcine coronary artery, with all branches ligated, mounted horizontally in a flow chamber; and (D) four flow chambers connected in parallel with mounted porcine coronary arteries.

staining using a standard methodology. Briefly, ring segments of PCA, approximately 2 mm in length, were sectioned and fixed in 10% formalin (361387P, VWR, UK) overnight. The segments were dehydrated in increasing concentrations of ethanol (EtOH), from 70% to 100%, and cleared in xylene substitute (Sigma-Aldrich A5597). Infiltration of paraffin (39503002, Paraplast X-TRA, McCormick Scientific) into the tissue occurred by immersing the segments for 2 hours at 60 °C. The segments were embedded in cassettes (720-0295, VWR, UK) with fresh paraffin and cooled at -20 °C. Cross-sections, 5 µm thick, were obtained using a Leica RM2125 RT microtome and mounted on slides for staining.

To process the slide-mounted sections for staining the slides were moved through xylene substitute, decreasing concentrations of EtOH, from 100% to 70%, and distilled water to remove paraffin and hydrate the sections. Subsequent to staining the slides were dehydrated through increasing concentrations of EtOH, from 95% to 100%, and cleared in xylene substitute. The sections were examined by light microscopy using an Olympus BX-51 microscope and images were obtained with an integrated Olympus DP50 camera. See Appendix A for detailed protocol.

3.1.3.1 Hematoxylin and Eosin Stain

Hematoxylin and eosin (H&E, GHS216 and HT110216, Sigma-Aldrich) stains the nucleus of a cell blue-black [124], therefore indicating the presence or absence of cells throughout the vascular wall. The sections were stained in hematoxylin and eosin for 10 minutes and 1 minute, respectively. See Appendix A for detailed protocol.

3.1.3.2 Modified Verhoeff Van Gieson Elastic Stain

Modified Verhoeff Van Gieson Elastic (HT-25A, Sigma-Aldrich) stains elastin blue-black to black. The slides were placed in Working Elastic Stain solution for 10 minutes, differentiated in Working Ferric Chloride Solution, and stained in Van Gieson Solution for 2 minutes.

3.1.3.3 Masson's Trichrome Stain

Masson's Trichrome (HT-15, Sigma-Aldrich) stains collagen blue and muscle fibres and cytoplasm red. The sections were treated overnight with Bouin's Solution, a mordant, at room temperature. The slides were then placed in working Weigert's iron hematoxylin solution, Biebrich scarlet-acid fuchsin, working phosphotungstic/phosphomolybdic acid solution, and aniline blue solution for 5 minutes each followed by 1% acetic acid for 2 minutes.

3.1.4 Immunocytochemical and Immunohistochemical Analysis

4',6-diamidino-2-phenylindole (DAPI) is a blue fluorescent nucleic acid stain that preferentially stains DNA [125]. Ring sections of non-decellularised and decellularised PCA were opened flat, fixed in 10% formalin overnight, and washed in phosphate buffered saline (PBS, P4417, Sigma-Aldrich) three times. The sections were stained with a 1/1000 dilution of DAPI (D9542, Sigma-Aldrich) for 15 minutes and washed three times in PBS. The luminal and abluminal surfaces were examined using a fluorescent microscope (Olympus BX-51) and images were taken using an integrated Olympus DP30BW camera.

Slide-mounted cross-sections of non-decellularised and decellularised PCAs were also stained with a 1/1000 dilution of DAPI for 1 minute to image nuclear material throughout the thickness of the tissue. In addition to the fluorescent images of the cross-sections, corresponding black and white images were obtained to identify the structure of the artery along with the nuclear material. This was beneficial for decellularised sections in particular as the arterial layers could be identified despite an absence of nuclear material. This was achieved by obtaining a snapshot image of the section using the Olympus DP30BW camera.

3.2 Results

Sections treated by agitation were analysed by DAPI staining of the luminal and abluminal surfaces. Figure 3.4 shows the effect of 0.1M NaOH treatment on the luminal surface with time. A high density of clearly defined circular nuclei was

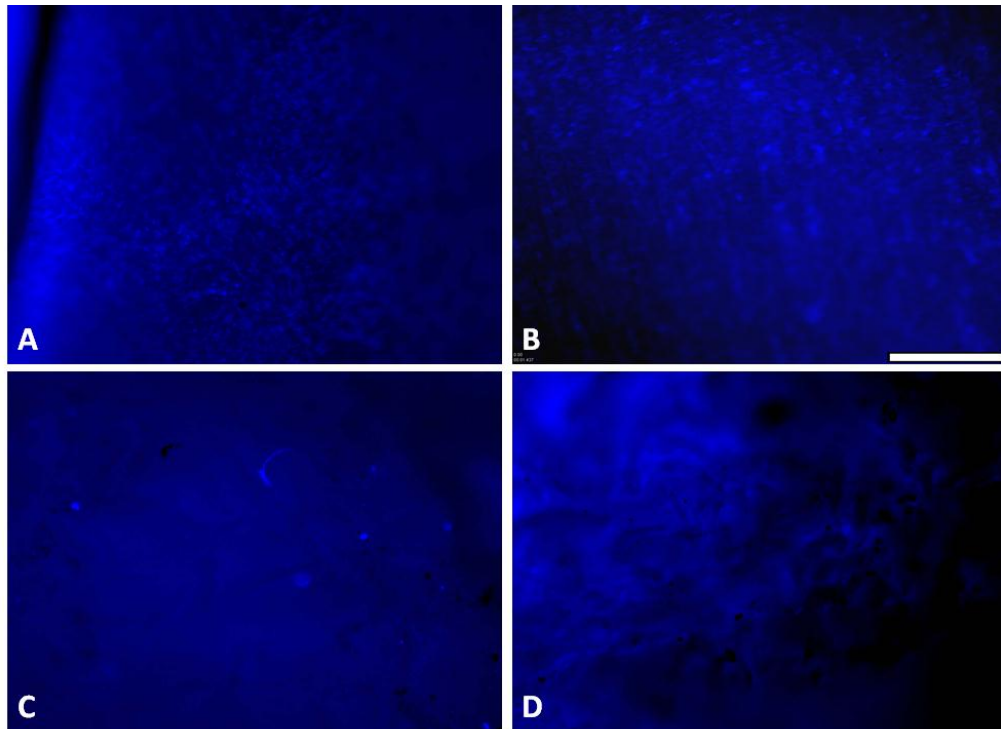


Figure 3.4: Effect of 0.1M NaOH treatment on porcine coronary arteries with time. DAPI staining of the luminal surface of flat sections of (A) 18 hours untreated porcine coronary artery and (B) 4 hours, (C) 12 hours, and (D) 18 hours agitation treated porcine coronary arteries. Scale bar: 200 μ m.

visible on the smooth surface of the untreated section. After 4 hours of 0.1M NaOH treatment (plus a further 3 hours in 0.9% saline) nuclei were still visible on the luminal surface, however, at a lower density and not as well defined. There were no cells visible after 12 hours and the surface was a smooth, continuous layer. However, after 18 hours treatment, in addition to no cells being visible, the pocketed appearance of the luminal surface suggested that significant damage of the underlying matrix had occurred, see Figure 3.4 (D). For a scaffold to perform as a viable TEBV it must be of a particular length with no branches, or at least with branches ligated. When flat 5 x 5 mm sections of PCA were decellularised for 9 hours, there were no cells visible throughout the scaffold as shown by H&E staining, see Figure 3.5 (B). However, the same protocol applied to a 40 mm length of PCA resulted in insufficient cell removal in central sections of the vessel, see Figure 3.5 (C). Therefore, decellularisation by perfusion was used as a more uniform method for cell removal, see Figure 3.5 (D).

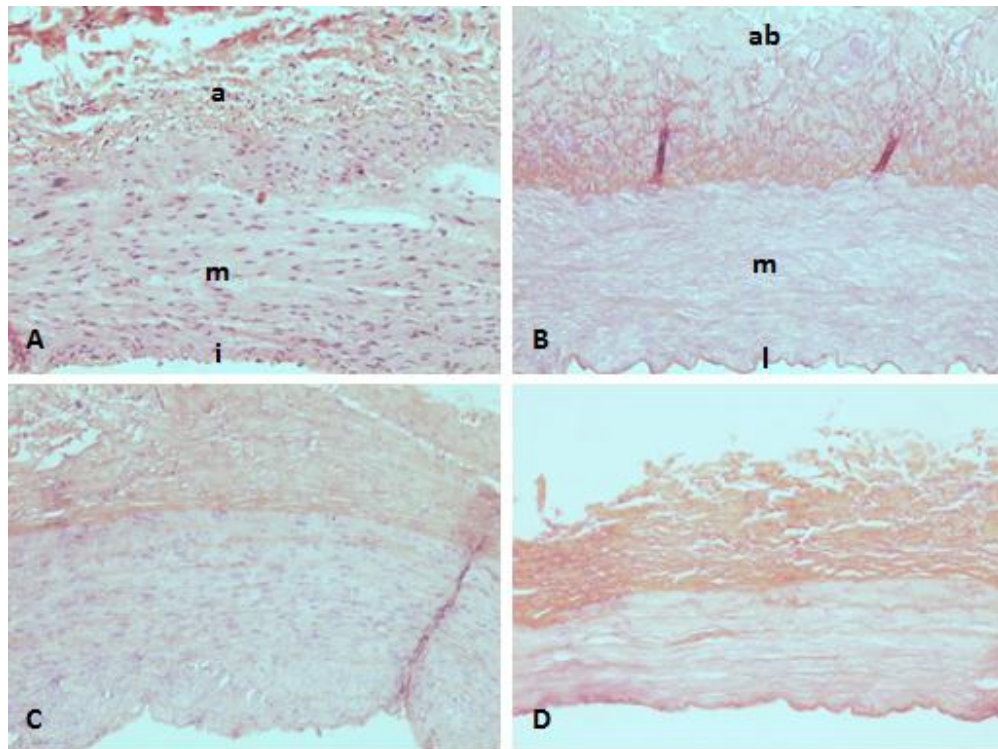


Figure 3.5: Effect of 0.1M NaOH treatment on porcine coronary arteries with time. DAPI staining of the luminal surface of flat sections of (A) 18 hours untreated porcine coronary artery and (B) 4 hours, (C) 12 hours, and (D) 18 hours agitation treated porcine coronary arteries. i: intima, m: media, a: adventitia, l: luminal surface, ab: abluminal surface. Magnification: 10x.

A combination of stains was used to determine the presence or absence of cells from the vascular wall and, therefore, the effectiveness of the decellularisation procedure. H&E staining of cross-sections of non-decellularised PCA clearly indicates the presence of cells throughout the vascular wall by the blue-black staining of the nuclei, see Figure 3.6. The compact medial layer and the looser adventitial layer of the vascular wall can be clearly distinguished in the images. The endothelial cells (ECs) of the intima, defined by their round nuclei, line the luminal surface of the vessel. The thick medial layer contains the elongated nuclei of vascular smooth muscle cells (vSMCs) orientated in the circumferential direction. The adventitia, the outer layer, contains the rounded nuclei of fibroblasts. There is no visible alteration in the cellular components of non-decellularised tissue following 9 hours in 0.9% saline, see Figure 3.6 (D–F). Figure 3.7 shows the progression of decellularisation with time. 0.1M NaOH treatment has an immediate effect on the cells of the vascular

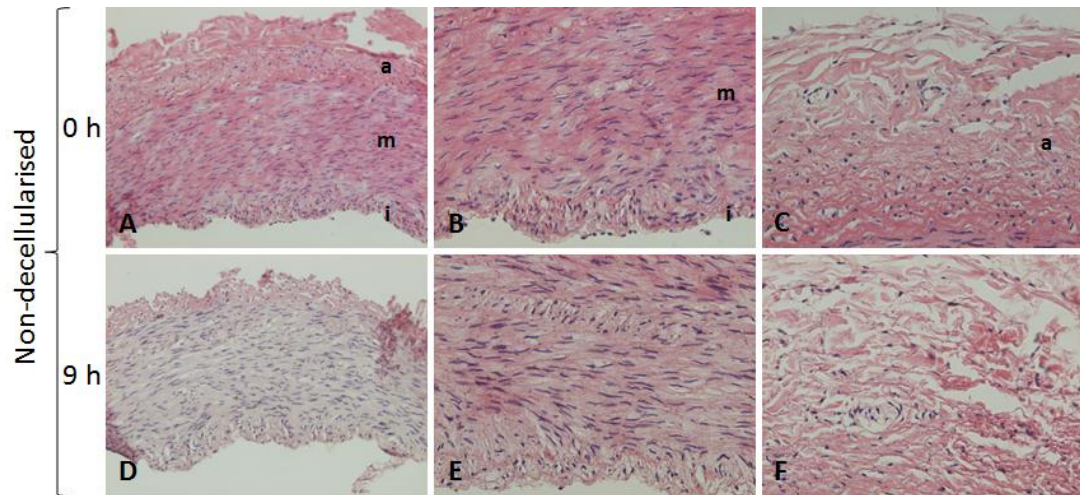


Figure 3.6: *H&E staining of non-decellularised porcine coronary arteries (A–C) immediately after excision and (D–F) after 9 hours in 0.9% saline. i: intima, m: media, a: adventitia. Magnification: (A, D) 10x, (B–C, E–F) 20x.*

wall as shown in Figure 3.7 (A–C). Cells are still visible throughout all layers however they are not as clearly defined as those of non-decellularised sections. After 8 hours decellularisation, the majority of the vessel wall is clear of nuclear material, however, there is a band of nuclei in the media near the external elastic lamina, Figure 3.7 (G–I). A decellularisation time of 9 hours or more was found to disrupt all cell nuclei, see Figure 3.7 (J–L). There is a loosening of both the medial and adventitial layers as a result of 0.1M NaOH treatment with increasing effect corresponding to increased treatment times. After 15 hours decellularisation larger pores are seen in both the media and adventitia, see Figure 3.7 (P–R).

DAPI staining of the cross-sections of non-decellularised and 0.1M NaOH treated PCA confirm the finding by H&E staining that cells are present within the vascular wall of non-decellularised and 6 hour treated PCA, see Figure 3.8 (A–F). There is no nuclear material visible after 9 hours of treatment, see Figure 3.8 (G–L). DAPI staining of the nucleic acid on the intimal and adventitial surfaces of non-decellularised PCA also shows the presence of cells across the surfaces, see Figure 3.9 (A–B). The size difference of the nuclei of ECs lining the luminal surface and the fibroblasts of the adventitia is evident. ECs form a uniform, single layer across the luminal surface while the fibroblasts are scattered across the abluminal surface. The adventitia is more difficult to image due to its porous, fibrous nature as

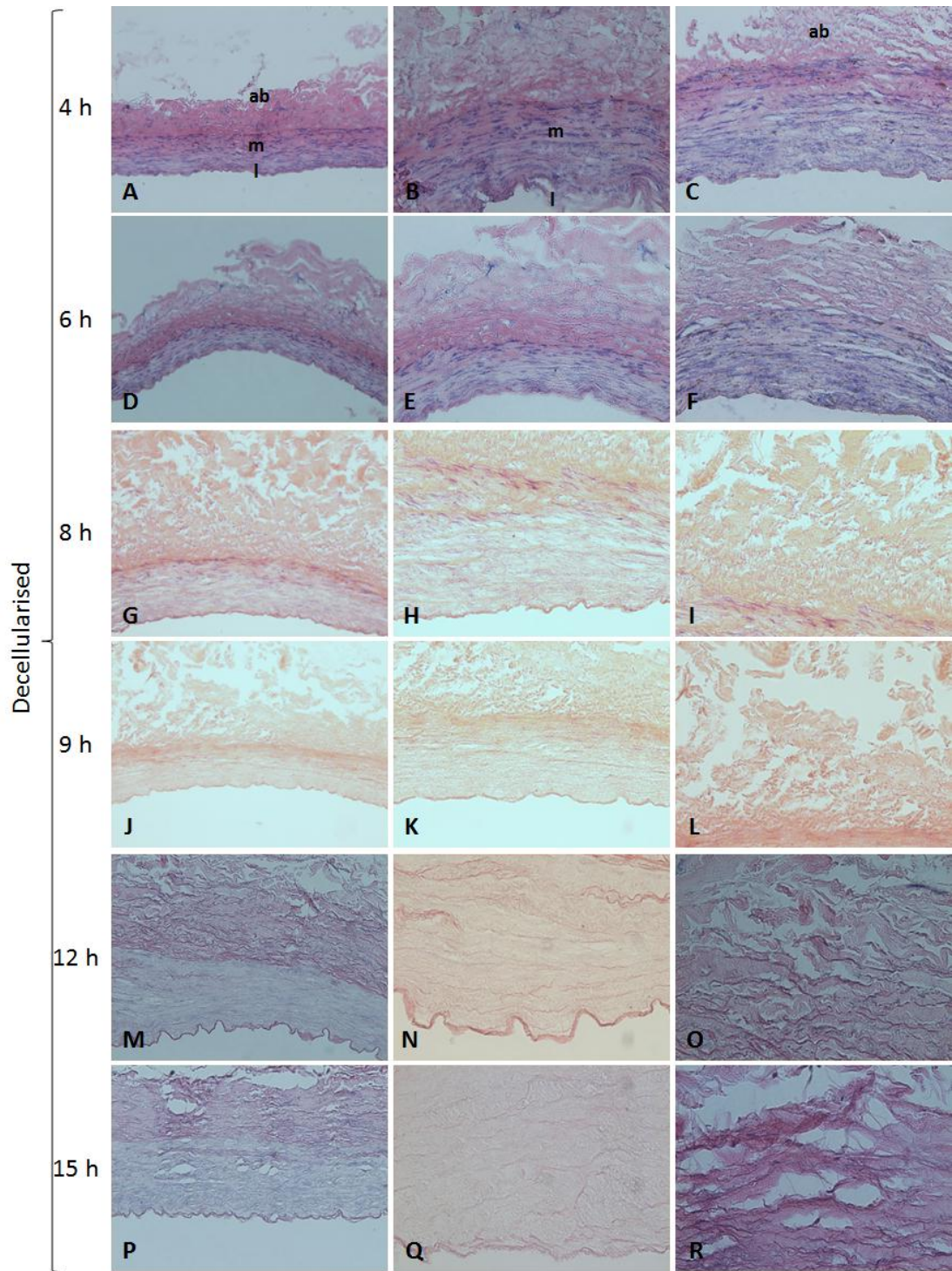


Figure 3.7: *H&E staining of the cross-sections of porcine coronary arteries treated with 0.1M NaOH by perfusion for between 1 and 12 hours and 0.9% saline for 3 hours. l: luminal surface, m: media, ab: abluminal surface. Magnification: (A, D, G, J, M, P) 10x, (B-C, E-F, H-I, K-L, N-O, Q-R) 20x.*

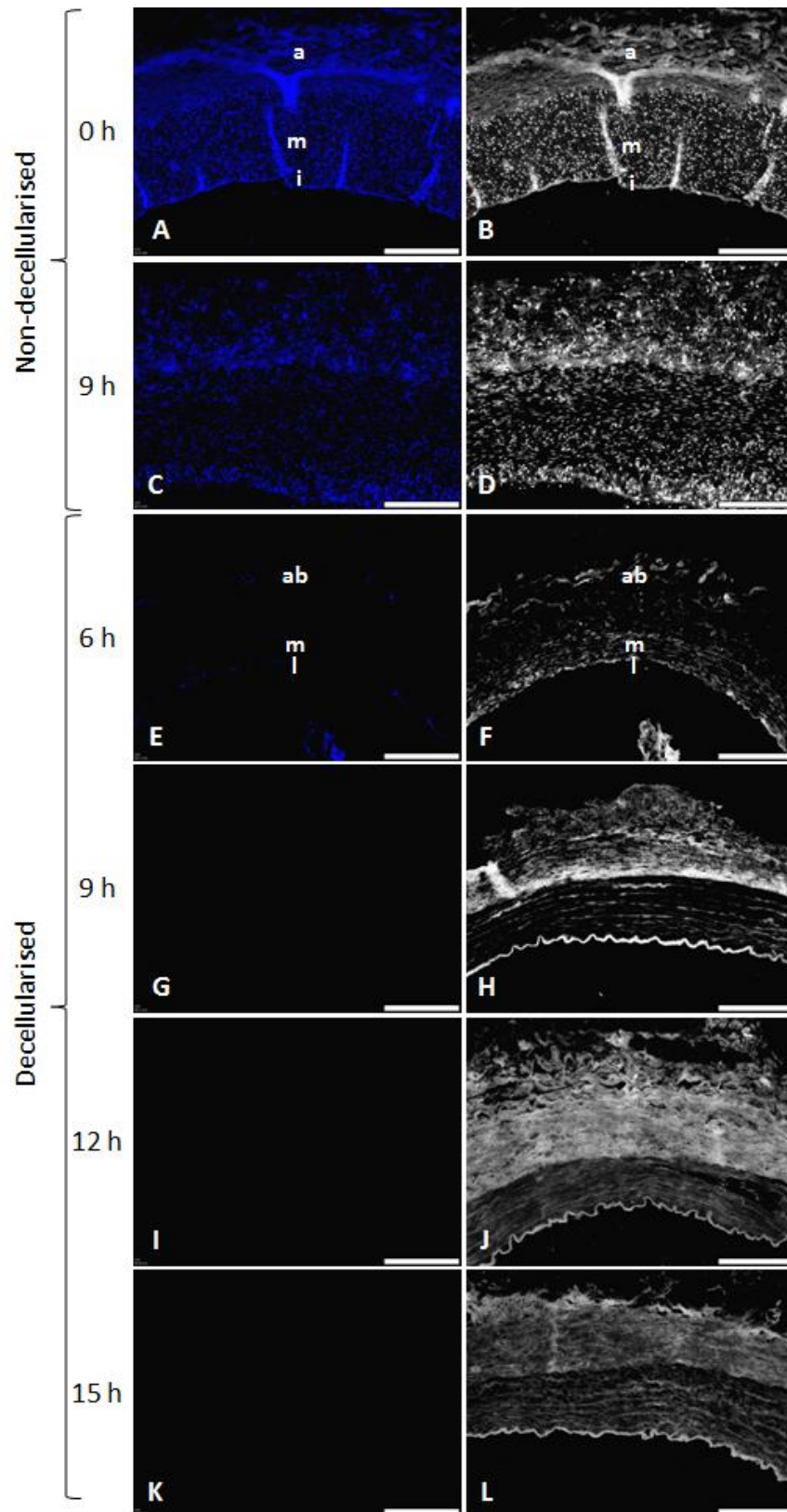


Figure 3.8: DAPI staining of the cross-sections of (A-D) non-decellularised and (E-L) 0.1M NaOH-treated porcine coronary arteries. *i*: intima, *m*: media, *a*: adventitia, *l*: luminal surface, *ab*: abluminal surface. Scale bar: 200 μm .

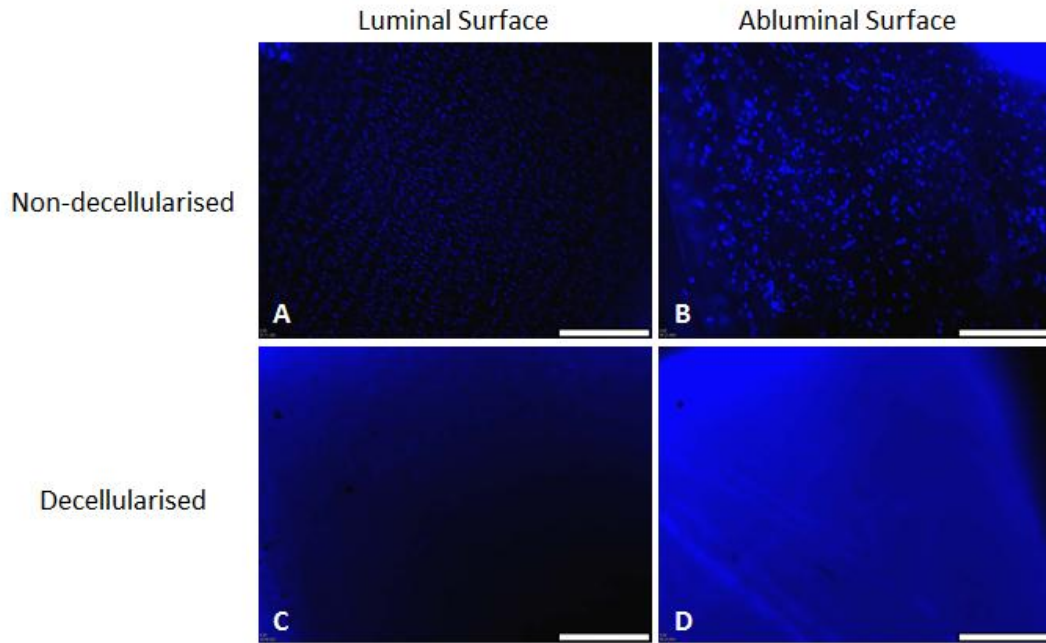


Figure 3.9: DAPI staining of the luminal and abluminal surfaces of (A-B) non-decellularised and (C-D) 9 hour decellularised porcine coronary arteries. Scale bar: 200 μ m.

opposed to the smooth, flat surface on the endothelium. After 9 hours perfusion decellularisation treatment, no cells are visible on either the luminal or abluminal surfaces of PCA, see Figure 3.9 (C-D).

Modified Verhoeff Van Gieson Elastic stain establishes the presence of elastin throughout the vascular wall by staining blue-black, see Figure 3.10. As expected with muscular arteries there are no bands of elastic laminae in the medial layer of the tissue although small amounts of elastin fibres are visible. The internal elastic lamina is prominent on the luminal side of all sections irrespective of decellularisation treatment. There are thick bundles of elastin fibres in the adventitia which loosen with increased decellularisation treatment times. Additionally, Modified Verhoeff Van Gieson elastic stains muscle yellow. This accounts for the yellow trace in the media of non-decellularised and 9 hour non-decellularised sections, see Figure 3.10 (A-F), while it is not visible in the treated sections. Masson's Trichrome stain highlights the presence of collagen throughout the vascular wall, staining blue, see Figure 3.11. Additionally, smooth muscle and cytoplasm stain red. While smooth muscle and cytoplasm are visible throughout the media of non-decellularised

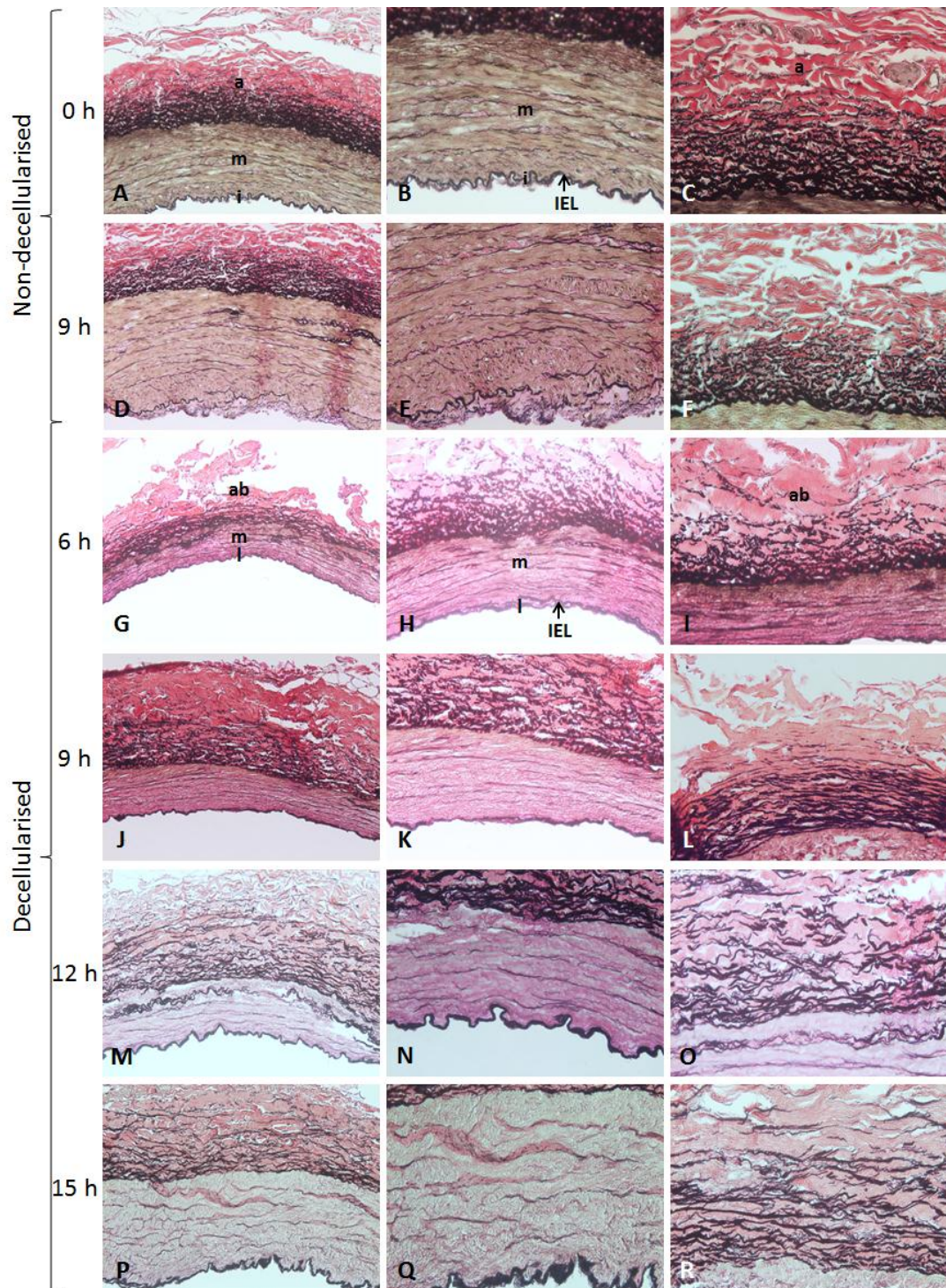


Figure 3.10: Modified Verhoeff Van Gieson Elastic staining of the cross-sections of (A-F) non-decellularised and (G-R) decellularised porcine coronary arteries. Elastin stains blue-black to black, collagen stains red. i: intima, m: media, a: adventitia, IEL: internal elastic lamina, l: luminal surface, ab: abluminal surface. Magnification: (A, D, G, J, M, P) 10x, (B-C, E-F, H-I, K-L, N-O, Q-R) 20x.

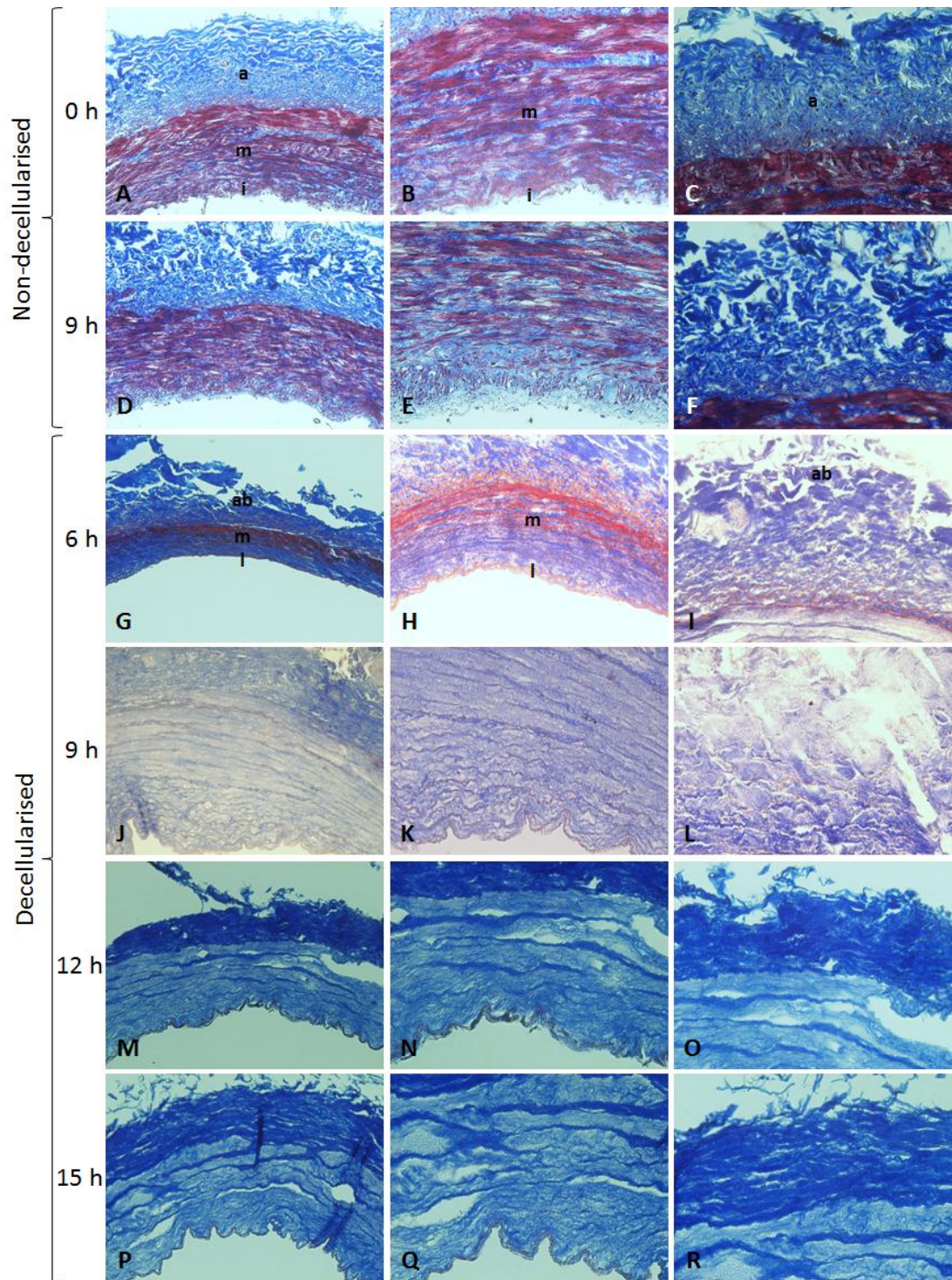


Figure 3.11: *Masson's Trichrome staining of the cross-sections of (A-F) non-decellularised and (G-R) decellularised porcine coronary arteries. Collagen stains blue, muscle fibres and cytoplasm stain red. i: intima, m: media, a: adventitia, l: luminal surface, ab: abluminal surface. Magnification: (A, D, G, J, M, P) 10x, (B-C, E-F, H-I, K-L, N-O, Q-R) 20x.*

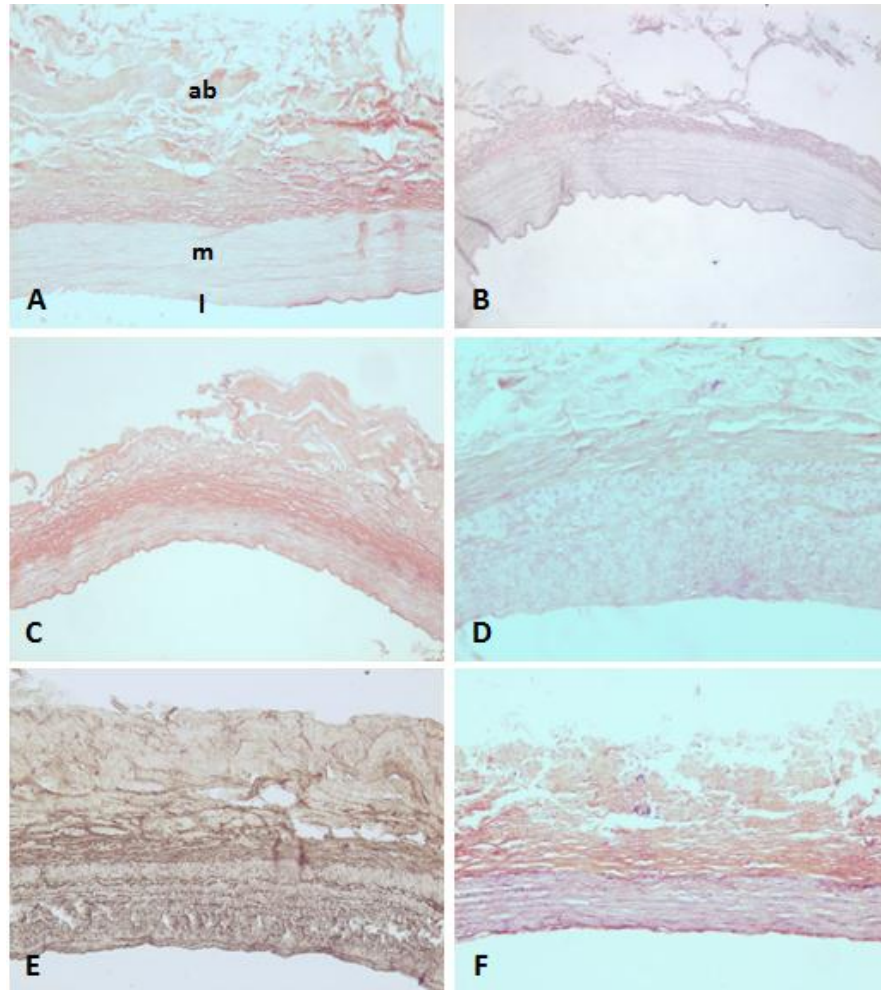


Figure 3.12: Thickness independence by perfusion decellularisation. H&E staining of the cross-section of six porcine coronary arteries of different thicknesses decellularised by perfusion for 9 hours. l: luminal surface, m: media, ab: abluminal surface. Magnification: 10x.

sections, there is partial removal after 6 hours treatment and total removal after 9 hours of treatment onwards. The denser collagen network of the adventitia is visible compared to the delicate collagen matrix of the media. This is also seen in the Modified Verhoeff Van Gieson elastic stain where collagen stains red.

The combination of these stains demonstrates that 9 hours decellularisation is an appropriate time period to effectively disrupt all the cells of the vascular wall producing a decellularised matrix. Additionally, perfusion decellularisation results in a process which is independent of the thickness of the artery, see Figure 3.12.

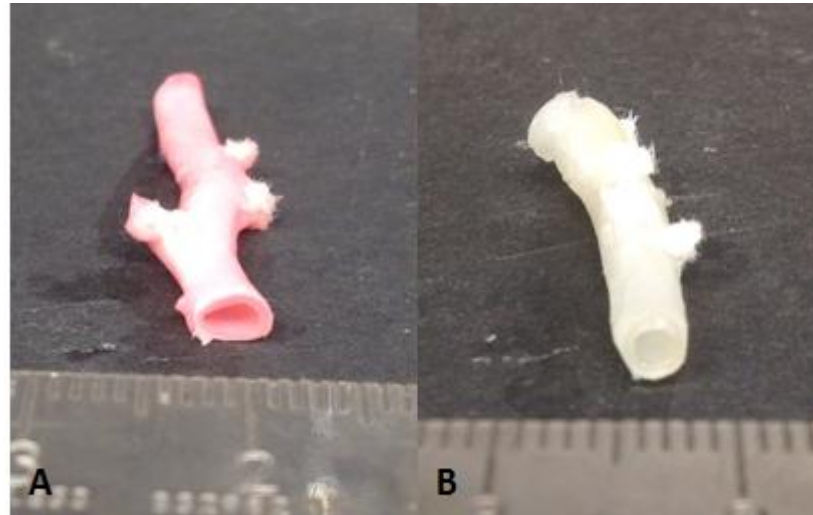


Figure 3.13: (A) *Non-decellularised* and (B) *9 hour decellularised porcine coronary arteries*.

Following 9 hours decellularisation, the artery is translucent compared to non-decellularised tissue, however it maintains its form, see Figure 3.13.

3.3 Discussion

Due to the wide range of species, blood vessels, timescales, and decellularisation protocols used it is difficult to determine the optimum approach to achieve a decellularised scaffold suitable for a TEBV. The overall aim is the development of a small-diameter TEBV with appropriate biomechanical and structural properties for procedures such as coronary artery bypass grafting (CABG). The aorta [10,100], carotid artery [92,99], femoral artery [91,102], and umbilical vein [55,108] among others have all been decellularised using various methods, yet these vessels are not structurally equivalent to the coronary arteries. While the structural characteristics of the coronary arteries are well documented [19], they have not previously been decellularised to achieve the coronary artery natural matrix scaffold. This is possibly due to their location in the cardiovascular system and the numerous branches extending from the main conduits, yet their potential as structurally equivalent vessels to act as bypass grafts for CABG is significant. In this study, an extensive and careful excision method was required to remove significant lengths of porcine

coronary arteries, up to 60 mm, with all branches ligated. The use of porcine vessels is justified due to the almost identical nature of porcine and human coronary circulation [33,34]. Some studies [65,91,92] remove the adventitial layer to aid the decellularisation process by providing direct reagent contact with the medial layer. These studies did not assess the effect of adventitial removal on the biomechanical properties of the vessels. However, the adventitia contains type I collagen bundles which offer structural support to the blood vessel. Removal of this layer would compromise the biomechanical properties of the resulting scaffold. Holzapfel et al. [20] performed uniaxial tensile tests on the three individual layers of human coronary arteries and determined the stress-stretch response of adventitia was stiffer than the media in the longitudinal direction while a similar response was found in the circumferential direction, see Figure 2.5 (page 12). This suggests that the removal of the stiffer adventitia could result in a more compliant vessel. As porcine coronary arteries have been found to be three times more elastic than human coronary arteries [13], a further increase in elasticity due to removal of the adventitia would be undesirable. The native architecture of the coronary arteries, consisting of the intima, media, and adventitia, is maintained in this study.

Short-term storage of soft tissue has demonstrated the natural degradation of tissue post mortem which results in an alteration of the biomechanical response [22,93]. After 48 hours storage at 4 °C, the initial slope of the stress-strain response of thoracic aortae significantly decreased (fresh vs. stored, 149.7 ± 19.7 kPa vs. 103.4 ± 13.2 kPa [93]) and the final slope significantly increased (fresh vs. stored, 925.5 ± 396.4 kPa vs. 1019.9 ± 445.1 kPa [93]). This highlights the need to minimise overall decellularisation time, even within 48 hours of harvesting, to maintain native biomechanical properties. However, the preservation of the extracellular matrix should not be compromised by more rapid decellularisation. The shortest decellularisation method described in the literature is of mouse aorta [103], significantly smaller than the coronary arteries used in this study. Tsai et al. agitated the tissue in 0.075% SDS for 2 hours followed by 100 minutes in PBS. However, significant degradation of the tissue as a result of the treatment is evident by delamination of the layers and high porosity of the matrix, see Figure 3.14. Liu et al. [76] achieved decellularisation of flat sections of rat aorta by agitation using 0.1M

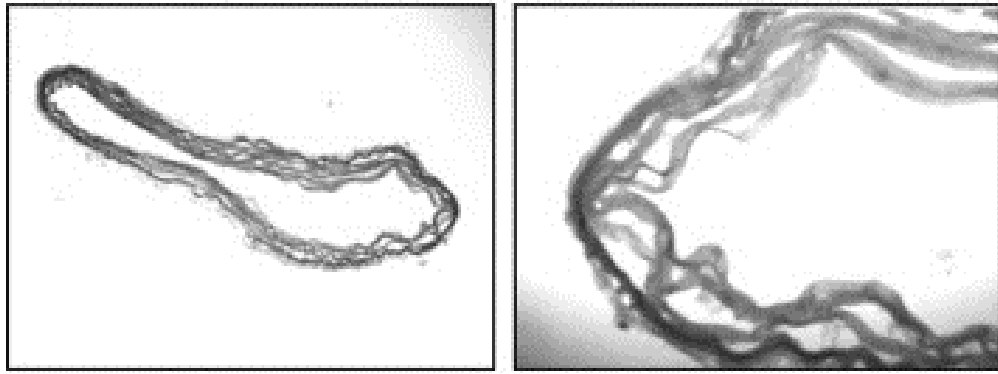


Figure 3.14: H&E staining of the cross-section of murine thoracic aorta decellularised with SDS for 2 hours and washed in PBS for 100 minutes (adapted from [103]).

NaOH and PBS over 4 hours without significantly disrupting the extracellular matrix. The tubular lengths of PCA natural matrix scaffold obtained in this study after 9 hours by perfusion is a considerable improvement compared to decellularisation of vessels of similar sizes [55,65,98].

The majority of decellularisation techniques use agitation to aid reagent infiltration of the vessel [10,29,36,55,91,95,100]. It is possible to decellularise short cylindrical and flat sections of the coronary arteries by agitation, see Figure 3.5, however, treatment by perfusion is necessary for decellularisation of full tubular lengths of arteries, with all branches ligated. Increased agitation treatment time may improve this outcome, although, as shown in Figure 3.4 (D), extended treatment causes significant damage to the remaining matrix. Additionally, Goissis et al. [94] found that increasing treatment time to improve cell removal had a significant impact on the collagen content of alkaline treated veins. Treatment by perfusion provided the most effective method for decellularisation of lengths of cylindrical segments, see Figure 3.5 and Figure 3.15. A study by Villegas Montoya and McFetridge [108] highlighted the improved decellularisation achieved by convective flow with transmural pressure removing cellular debris from the tissue. SEM analysis showed that cells remained in the medial layer of agitated sections but not of perfused sections.

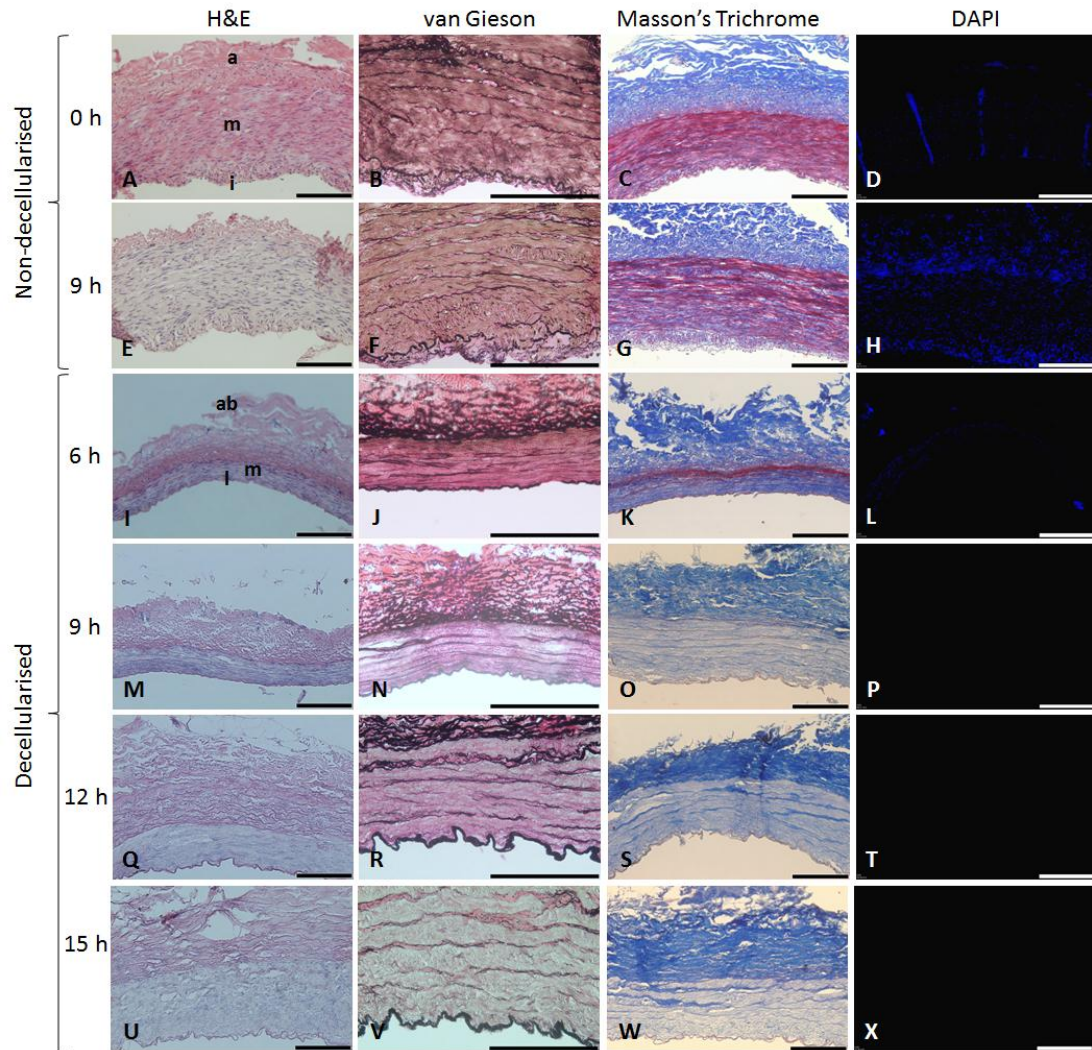


Figure 3.15: Summary of the effect of NaOH treatment on porcine coronary arteries. (A, E, I, M, Q, U) H&E, (B, F, J, N, R, V) Verhoeff van Gieson Elastic, (C, G, K, O, S, W) Masson's Trichrome, and (D, H, L, P, T, X) DAPI staining the cross-sections of (A-D) non-decellularised PCA, (E-H) 9 hour non-decellularised PCA, and PCA decellularised for (I-L) 6 hours, (M-P) 9 hours, (Q-T) 12 hours, and (U-X) 15 hours. *i*: intima, *m*: media, *a*: adventitia, *l*: luminal surface, *ab*: abluminal surface. Scale bar: 200 μ m.

A summary of the histological and immunohistochemical analysis used to verify decellularisation of PCA is shown in Figure 3.15. Non-decellularised segments of PCA stained with H&E and DAPI clearly show the presence of cell nuclei throughout the vascular wall, see Figure 3.15 (A, D-E, H). H&E, DAPI, and

Masson's Trichrome staining of 6 hour decellularised sections all indicate that intact cells remain in the outer portion of the medial layer, see Figure 3.15 (I, K-L). Following 9 hour decellularisation no intact cell nuclei or smooth muscle content was visible throughout the vascular wall, see Figure 3.15 (M, O-P). Further treatment resulted in scaffolds with increased porosity which could aid in the repopulation of the scaffold. Modified Verhoeff Van Gieson Elastic stain and Masson's Trichrome stain show the collagen and elastin matrices in the medial and adventitial layers. The media of muscular arteries consists mainly of vSMCs with a delicate interlaced matrix of collagen and elastin [19]. The adventitia on the other hand consists of thick collagen bundles and elastic fibres. These differing compositions are demonstrated by darker staining of the adventitia compared to the media for both Modified Verhoeff Van Gieson Elastic stain and Masson's Trichrome stain, see Figure 3.15 (B-C, F-G, J-K, N-O, R-S, V-W). Staining of decellularised sections does not indicate a degradation of the collagen and elastin matrix, however, there appears to be a loosening of the fibres, in particular in the elastic fibres in the adventitia of 15 hour decellularised sections, see Figure 3.10 (P, R). The internal elastic lamina has been preserved in all decellularised sections which is vital for this scaffold to perform as a TEBV scaffold or graft. The internal elastic lamina exhibits inflammation- and thrombosis-resistant properties compared to collagen-based blood contacting materials [76]. Additionally, the internal elastic lamina acts as a barrier to prevent smooth muscle cells migrating to the intima resulting in intimal hyperplasia as well as providing an appropriate surface for endothelial cell attachment [107].

Degradation of the collagen content of decellularised tissue with NaOH has been used to increase the porosity of decellularised tissue for improved cell infiltration [92]. In that study, an enzymatic/detergent decellularisation treatment, which successfully removed all vascular cells, caused a degree of disruption to the extracellular matrix. NaOH (0.5M) was used to further degrade the matrix of porcine carotid arteries over 2 hours, removing small collagen fibres. An additional treatment of 0.1M NaOH for 24 hours further disrupted the collagen network, in particular the collagen fibres bundles, resulting in a highly porous matrix. In this current study, the use of a lower concentration of NaOH (0.1M) in isolation for a shorter overall time has resulted in a matrix which, based on histological analysis, maintains its collagen

content, see Figure 3.10 and Figure 3.11. The preservation of collagen will be further investigated in Chapter 4 by an examination of the biomechanical properties.

Muscular porcine coronary arteries, structurally equivalent to human coronary arteries, have been successfully decellularised within 9 hours limiting the natural degradation of the tissue and maintaining the gross extracellular matrix characteristics. This short-term decellularisation method achieving the natural matrix scaffold is considerably shorter than other decellularisation methods in the literature for vessels of its size. A short-term decellularisation method is clearly more commercially viable than longer term decellularisation protocols. Additionally, through the use of perfusion decellularisation, appropriate lengths of PCA scaffold suitable for use as grafts have been obtained. Through biomechanical analysis of non-decellularised and decellularised arteries, in Chapter 4 it will be determined whether NaOH results purely in the removal of vascular cells or additionally disrupts extracellular matrix components. While complete decellularisation has been achieved after 9 hours treatment, the increased porosity observed in sections with extended treatment times may aid in the repopulation of the scaffold, however, this may also compromise the biomechanical properties. In addition, achieving the natural matrix scaffold of coronary arteries could provide vital benchmarking information on the development of other grafts for use in CABG in terms of structural and biomechanical properties.

Chapter 4 Biomechanical Evaluation of Non-decellularised and Decellularised Porcine Coronary Arteries

Despite the prevalence of coronary artery disease and the requirement for surgical interventions such as balloon angioplasty, stenting, and coronary artery bypass grafting (CABG) there is limited analysis of the biomechanical properties of the small-diameter muscular coronary arteries [13,20,21,30,126]. Furthermore, there are no studies of the biomechanical properties of the coronary artery natural matrix scaffold. As described in Chapter 3, a short-term decellularisation method was developed to successfully obtain the natural matrix scaffold of porcine coronary arteries (PCA). Histological analysis confirmed the removal of vascular cells and the preservation of elastin and collagen in the decellularised matrix. To determine whether the elastin and collagen content has been affected as a result of the decellularisation procedure the biomechanical properties of non-decellularised and decellularised PCAs are evaluated. The short-term (< 48 hours) alteration in mechanical properties of arterial tissue has previously been reported [22,93]. Therefore, to evaluate the short-term natural degradation as a result of harvesting, 9 hour non-decellularised PCAs are also analysed. Additionally, the capability of the natural matrix scaffold to act as a TEBV graft or scaffold is assessed. A comprehensive understanding of both the native blood vessel and natural matrix

scaffold can inform the development of other synthetic and natural grafts for TEBV scaffolds.

By conducting uniaxial tensile, inflation, and permeability tests and a dimensional analysis this chapter aims to:

- Characterise the biomechanical properties of non-decellularised PCA immediately upon excision;
- Determine the effect of 0.1M NaOH treatment on the biomechanical properties of decellularised PCA;
- Determine the short-term natural degradation of non-decellularised PCA corresponding to decellularisation time;
- Evaluate the potential of the natural matrix scaffold to act as a biomechanically viable graft or vascular tissue engineering scaffold.

4.1 Materials and Methods

4.1.1 Tissue Harvesting and Preparation

Forty-five porcine hearts, weighing an average of 390 g, were obtained for biomechanical tests. Arteries were prepared and excised from porcine hearts as described in Section 3.1.1 (pg. 44). Additional lengths of PCA were excised without the branches ligated for non-decellularised permeability and uniaxial tensile tests. The breakdown of biomechanical tests performed on individual PCAs is shown in Figure 4.1. The variability in the mechanical response of the main coronary arteries from the same heart was found to be lower than the variability between individuals [13]. Therefore, right coronary arteries (RCA), left anterior descending coronary arteries (LAD), and left circumflex coronary arteries (LCX) were obtained for uniaxial tensile, inflation, and permeability tests, respectively. A dimensional analysis was carried out on all three blood vessels. PCAs were decellularised by perfusion for either 9 hours or 15 hours as described in Section 3.1.2 (pg. 45). Test segments were stored in 0.9% saline at room temperature prior to testing. All tests were performed within 24 hours of slaughter to minimise the effect of degradation as

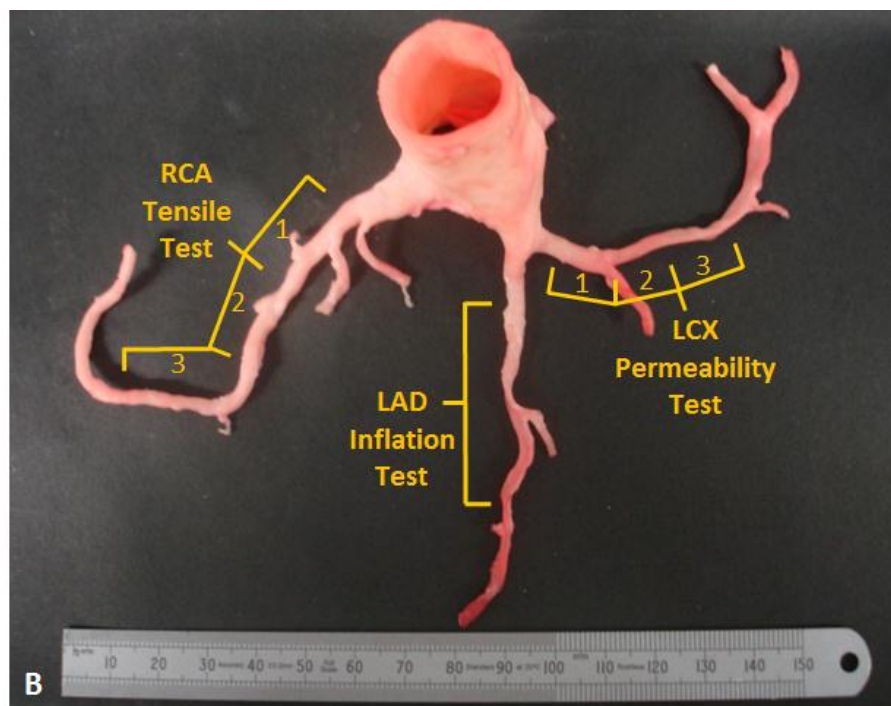
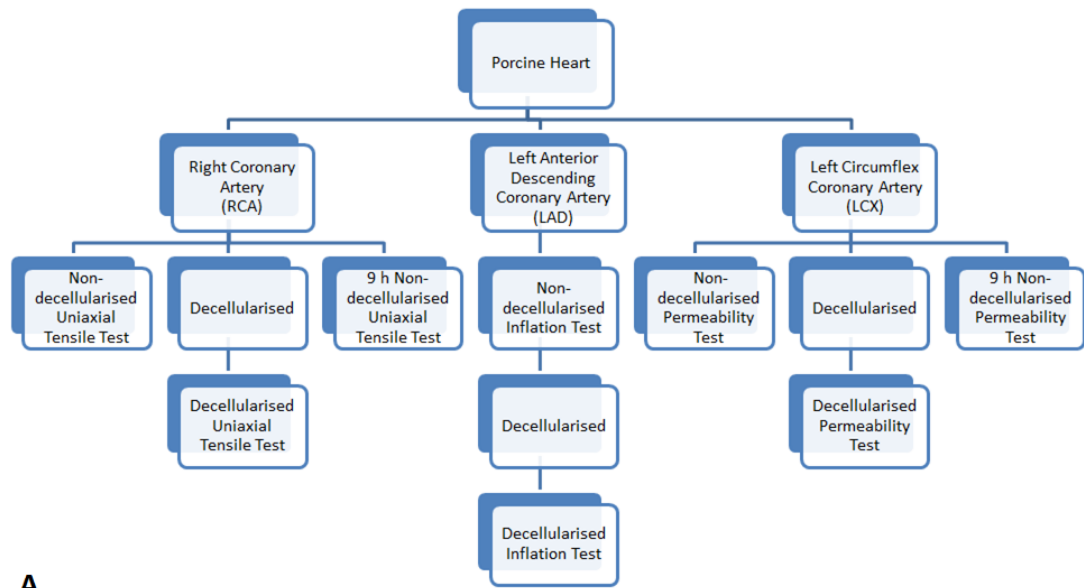


Figure 4.1: Biomechanical tests performed on each porcine coronary artery. (A) Flow chart and (B) illustration detailing the biomechanical tests performed on segments of the three main coronary vessels.

a result of harvesting. Unsuccessful tests were a result of unsuitable diameters or lengths of PCA or branches located in the test region.

4.1.2 Uniaxial Tensile Tests

Uniaxial tensile tests in the longitudinal direction were performed to determine the stress-strain response of non-decellularised PCAs and the corresponding alteration as a result of decellularisation. Twenty-six RCAs were divided into segments 20 mm in length. Depending on appropriate lengths and diameters, where possible, up to three segments were obtained from each RCA, see Figure 4.1 (B). Non-decellularised (N_0 , $n=25$), 9 hour non-decellularised (N_9 , $n=18$), and 9 hour decellularised (D_9 , $n=19$) sections were tested.

Segments were opened in the longitudinal direction and dog-bone shaped sections, with dimensions of 3.7 mm width and 12 mm gauge length, were obtained using a custom-made die, see Figure 4.2 (A). Sections containing branches within the gauge length were discarded. The sections were mounted into custom-made grips and attached to a 20 N load cell within a Zwick Z005 displacement controlled tensile testing machine (Zwick GmbH, Ulm, Germany), see Figure 4.2 (B). The grip faces were lined with sand paper to prevent tissue slippage during loading. The sections were pre-conditioned to reach a steady state and allow for comparison [25]. This was performed at a strain rate of 60 %/min of the initial gauge length measured at a pre-load of 0.001 N and to a maximum force of 1 N over 5 cycles, see Figure 4.2 (C). Sections were irrigated during pre-conditioning with 0.9% saline. Sections were then tested to failure at a strain rate of 60 %/min of the initial gauge length. Failure occurred when the section began to tear at either grip or along its length.

The force-extension data recorded by the Zwick Z005 was converted to stress-strain for comparison of the sections. Stress was calculated as:

$$\sigma = \frac{F}{A_0} \quad \text{MPa} \quad (\text{Equation 4.1})$$

where, F is the instantaneous force (N) and A_0 is the original cross-sectional area (m^2). The gauge length thickness was required to calculate the cross-sectional area. Sections of RCA directly adjacent to the test section were obtained and the thickness was measured using a microscope with an integrated linear micrometer (Mitutoya), see Figure 4.2 (D). An average of six measurements was taken as the section thickness.

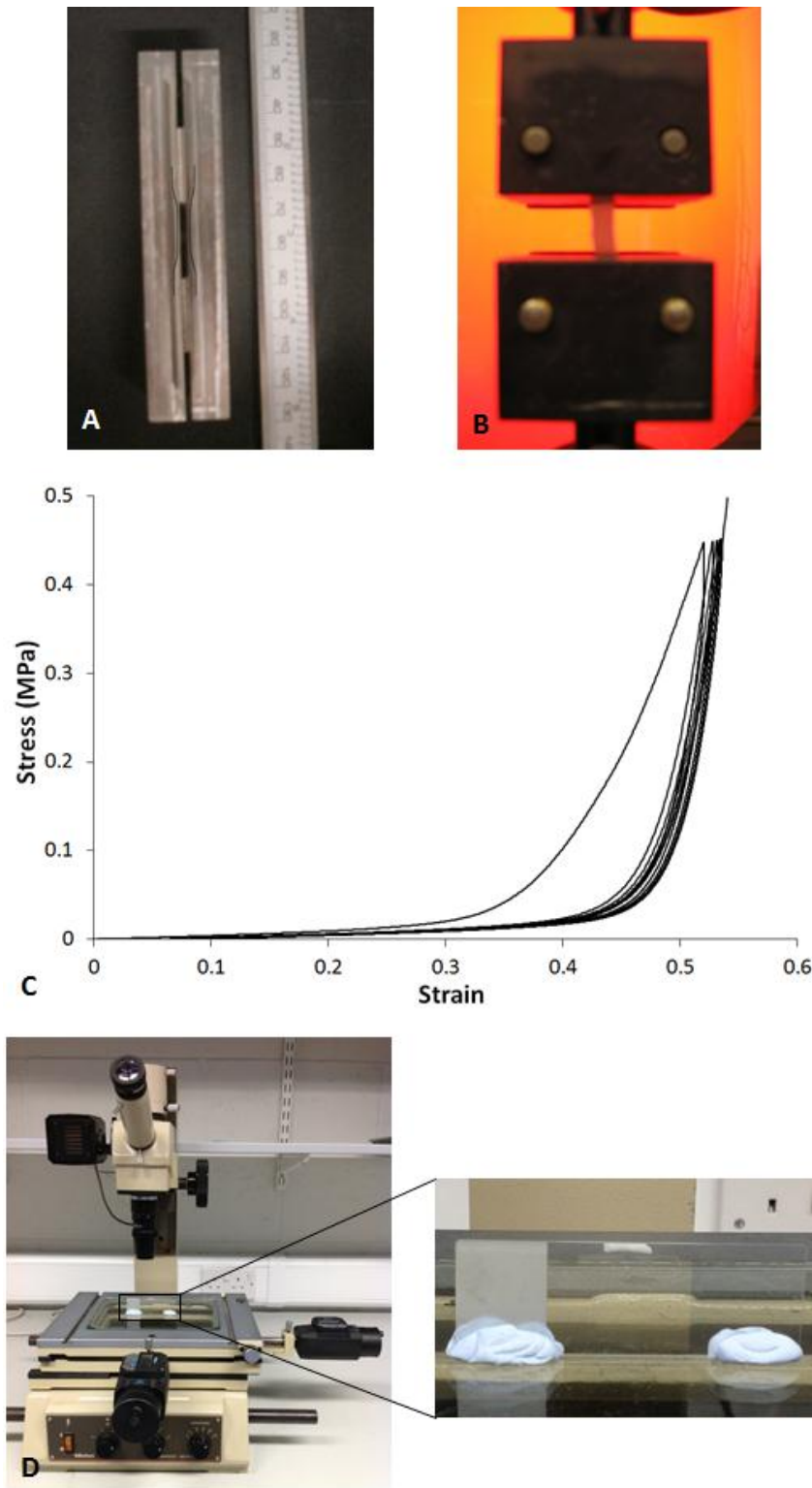


Figure 4.2: Uniaxial tensile test set-up. (A) Dog-bone shaped die to obtain sections; (B) right coronary artery section mounted in custom-made grips within the Zwick Z005 displacement controlled tensile testing machine; (C) typical pre-conditioning curve over 5 cycles to a maximum force of 1 N; and (D) slide-mounted section to measure thickness.

Strain was calculated as:

$$\varepsilon = \frac{\Delta l}{l_0} \quad (\text{Equation 4.2})$$

where, Δl is the change in length (m) and l_0 is the original length (m) taken at a pre-load of 0.001 N after pre-conditioning.

Further uniaxial tensile tests were performed on sections decellularised for 15 hours (D_{15} , $n=6$) to investigate the effect of increased treatment time on the biomechanical properties. The tests were performed as described above.

4.1.3 Inflation Tests

Inflation tests were performed to determine the response of intact PCA under physiological pressures and the corresponding change as a result of decellularisation. Seven segments of LAD, approximately 40 mm in length and with all branches ligated, were obtained, see Figure 4.1 (B). Each vessel was cannulated at both ends with natural polypropylene barbs (MTLL004-6, Value Plastics, CO, USA) and mounted horizontally into a custom-made flow chamber, see Figure 4.3 (A). A pre-stretch of 30% was applied to the artery using a linear micrometer attached to the flow chamber to imitate in vivo conditions [13,36]. The flow chamber was connected to a pressure head of 0.9% saline, the height of which was altered using a pulley system to achieve pressures of between 10 mmHg and 200 mmHg, see Figure 4.3 (B). The luminal pressure was measured using a paediatric pressure catheter (Abbott, IL, USA) connected to a pressure transducer (DTXPlus, BD Medical Systems, NJ, USA), amplifier and monitoring device (TA-100, CWE Inc., PA, USA). Five pre-conditioning cycles of 10 - 200 mmHg were applied to remove the effect of hysteresis and allow for repeatability across the test segments, see Figure 4.4. The LAD was then inflated from 10 - 200 mmHg in increments of 10 mmHg. At each step a video extensometer (MESSPHYSIK Material Testing, Austria) immediately recorded an image. The vessel was discarded if substantial leaks developed during the test. The flow chamber was interchangeable between the inflation and the perfusion decellularisation set-ups. The LAD was decellularised for 9 hours after which it was immersed in 0.9% saline for 30 minutes to allow it recover

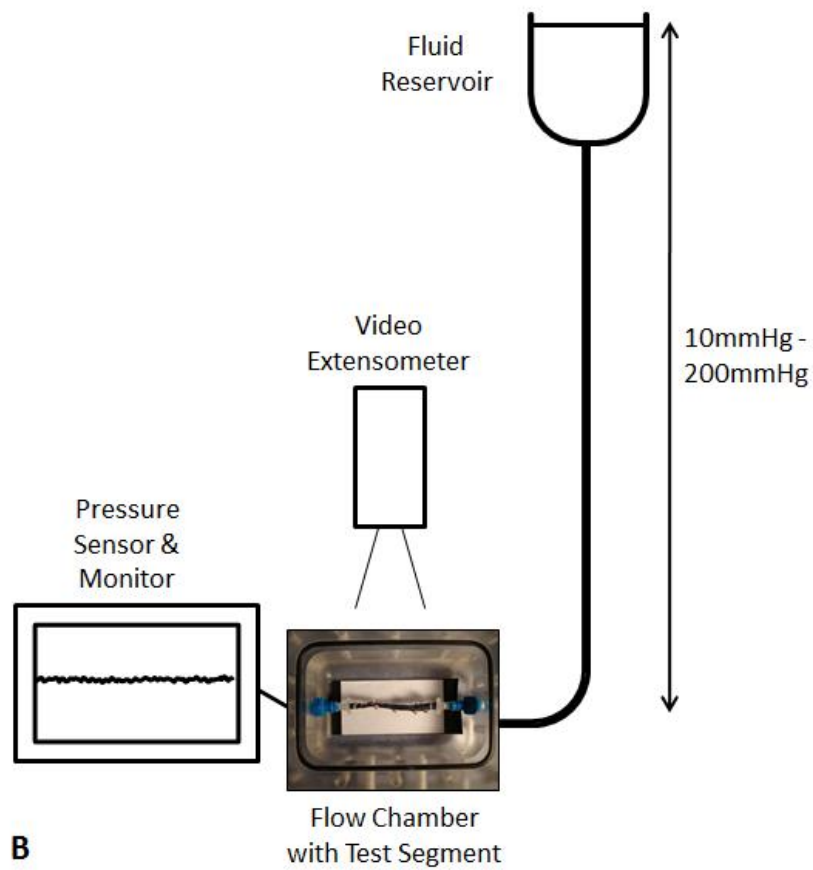
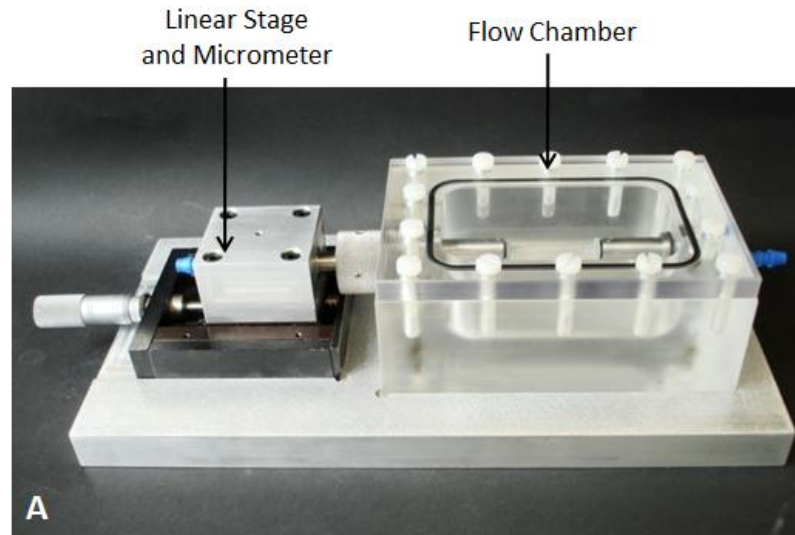


Figure 4.3: Inflation test set-up. (A) Flow chamber where a precise pre-stretch is applied to the horizontally mounted artery using the linear micrometer. **(B)** Schematic of the inflation test set-up where the height of the reservoir is controlled by a pulley system and the mounted artery is monitored by a pressure sensor and video extensometer.

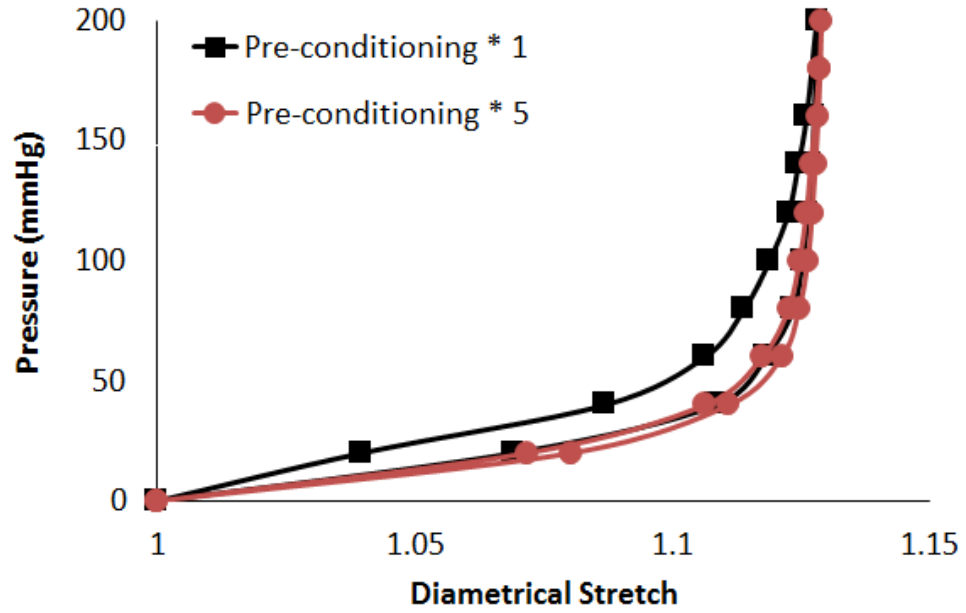


Figure 4.4: *Pre-conditioning for inflation tests of the left anterior descending coronary artery over 5 cycles to reduce the effect of hysteresis.*

its relaxed state. The inflation test was then repeated under identical conditions to those outlined above. Image analysis of the video extensometer images at each time step resulted in multiple measurements of the external diameter along the length of the LAD. Combined with the data obtained from the pressure sensor, external diameter-pressure curves and pressure-diametrical stretch curves were obtained. Diametrical stretch was calculated as:

$$\text{Diametrical Stretch} = \frac{\phi}{\phi_0} \quad (\text{Equation 4.3})$$

where, ϕ is the instantaneous diameter (mm) and ϕ_0 is the original diameter (mm) at 10 mmHg.

4.1.4 Permeability Tests

Permeability tests were performed to determine the alteration as a result of decellularisation, and also to determine whether decellularised PCA can maintain the ability to withstand high pressures. Twenty-one LCXs were divided into segments, each 15 mm in length, and were tested non-decellularised (N_0 , $n=17$), 9 hour non-decellularised (N_9 , $n=10$) or 9 hour decellularised (D_9 , $n=11$), see Figure 4.1 (B).

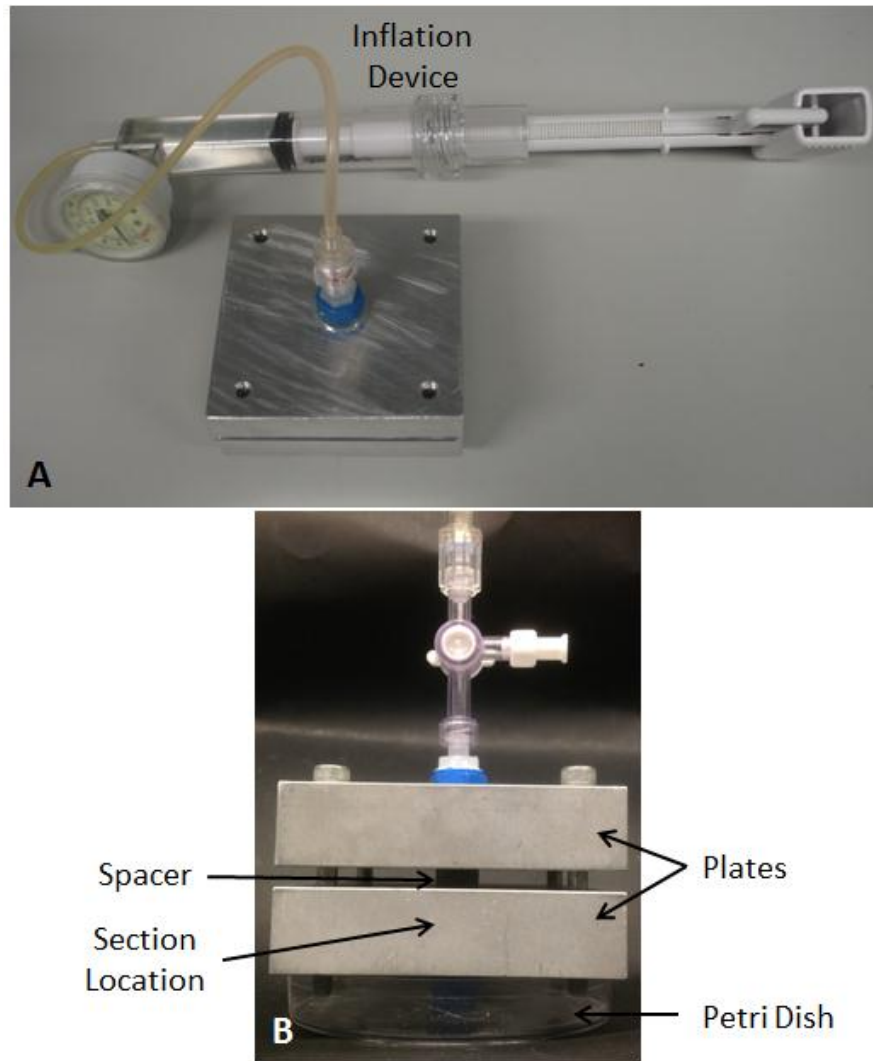


Figure 4.5: Permeability test set-up. (A) Inflation device attached to permeability rig; and (B) close-up of device.

The segments were opened in the longitudinal direction and 8 mm circular sections were obtained using a custom-made die. Sections containing branches were discarded.

The permeability rig consists of two plates with a 4 mm hole through the centre of both. The top plate secures to a bottom plate by four screws, see Figure 4.5. There is a counterbore in the facing surfaces of each plate, 8 mm in diameter and 5 mm deep. A spacer with an external diameter of 8 mm and an internal diameter of 4 mm sits in the top well. The LCX section was placed in the well of the bottom plate obstructing the 4 mm central hole. A water-filled inflation device (Basix 25, Merit Medical

Systems, UT, USA), connected to the top plate through a luer connection, increased the pressure to 1500 mmHg for 60 minutes. A petri dish under the bottom plate collected fluid perfused through the section for the duration of the test. The petri dish was weighed before and after the test using a digital Mettler AE50 balance, accurate to 0.1 mg. The difference in mass was that of the fluid which passed through the 4 mm diameter section of PCA exposed to the pressurised water over the course of 60 minutes. Permeability was calculated using Darcy's Law:

$$k = \frac{QL\mu}{A\Delta P} \quad \text{m}^2 \quad (\text{Equation 4.4})$$

where, k is the permeability coefficient (m^2), Q is flow rate (m^3/s), L is tissue thickness (m), μ is viscosity of water ($\text{Pa}\cdot\text{s}$), A is surface area (m^2), and ΔP is pressure drop across the material. The thickness was measured from sections of LCX directly adjacent to the test section using a microscope with an integrated linear micrometer. An average of six measurements was obtained.

4.1.5 Dimensional Analysis

The removal of cells from the vascular wall and the potential damage to the extracellular matrix (ECM) may alter the dimensions of the vessel. Fourteen non-decellularised sections of PCA and fourteen corresponding 9 hour decellularised sections of PCA were obtained, each 2 mm in length. The sections were taken from regions of PCA as close together as possible. Images were taken of the intact (no-load state) cross-section under a microscope with an integrated camera, see Figure 4.6 (A). The sections were cut in the longitudinal direction (zero-stress state) and a second image was immediately taken, see Figure 4.6 (B). The images were analysed for measurements of thickness, internal and external circumference, and opening angle using ImageJ 1.42 software (National Institutes of Health, MD, USA), see Figure 4.6 (C-D). The opening angle (Θ) was calculated by measuring the angle from the centre of the internal circumference to the outer edges of open sections [29,30], see Figure 4.6 (D).

Additionally, the pre-stretch of ten LADs was measured. The artery was marked in two locations in situ and the length measured using a vernier callipers. Upon excision

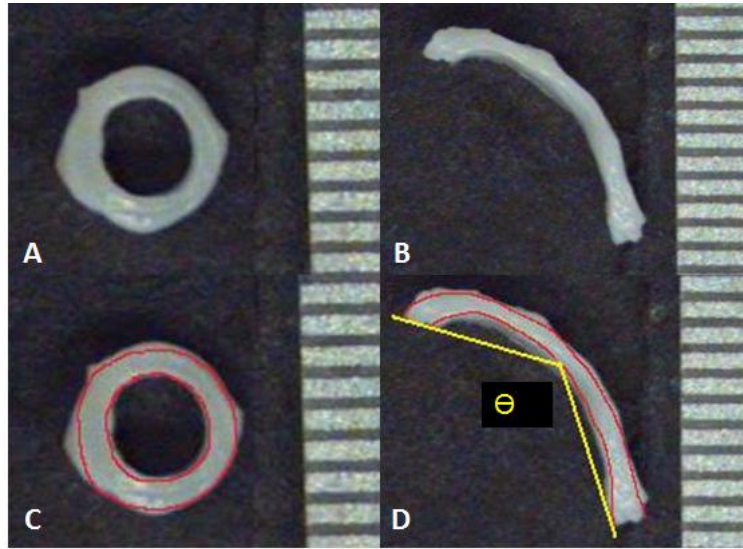


Figure 4.6: Dimensional analysis. Microscope images of sections of non-decellularised PCA in the (A) no-load and (B) zero-stress state with precision ruler for calibration; and measurement of (C) intact internal and external circumferences and (D) open internal and external circumferences and opening angle.

and arterial recoil, the length was measured again and the pre-stretch was calculated as:

$$\lambda = \frac{L_i}{L_e} \quad (\text{Equation 4.5})$$

where, L_i is the in situ length and L_e is the ex situ length.

4.1.6 Statistics

All data is presented as mean $\pm$ standard deviation (SD). Error bars indicate one standard deviation unless otherwise stated. D'Agostino and Pearson omnibus K2 normality tests were performed using GraphPad Prism version 5 for Windows (GraphPad Software, CA, USA). In some instances the removal of outliers was required to achieve normality. Outliers were defined as data points outside the mean $\pm$ 2SD. Using Microsoft Excel (2010, Microsoft, WA, USA), F-tests were performed on data sets to determine equal or unequal variance prior to t-tests to ascertain whether the means of two data sets are statistically different from each other. A p value < 0.05 was considered significant.

4.2 Results

4.2.1 Uniaxial Tensile Tests

All sections exhibited the typical non-linear J-shaped curve associated with soft tissues for representative stress-strain responses, see Figure 4.7. The variability in the elastic behaviour was evident for all groupings. The slope of the curve was analysed in three regions; the initial slope, the pre-conditioned slope, and the final slope; by a linear fit of the data in that region, see Figure 4.8 (B). Additionally, the x-intercepts for the pre-conditioned and final slopes were also analysed as a measure of the distensibility of the artery.

The 9 hour decellularised sections exhibited less stiff behaviour in the initial linear region but statistically significant stiffer behaviour in both the pre-conditioned and final regions of the curve when compared to non-decellularised and 9 hour non-decellularised sections, see Figure 4.9 and Table 4.1. The x-intercepts for the pre-conditioned and final slopes occur at a statistically significant higher strain for 9 hour decellularised sections than for non-decellularised or 9 hour non-decellularised sections, see Figure 4.9 and Table 4.1. The gauge length of all sections prior to pre-conditioning are similar; however, after pre-conditioning the gauge length of the decellularised sections does not extend to the same degree as the non-decellularised and 9 hour non-decellularised sections. Finally, the thickness of the non-decellularised and 9 hour non-decellularised sections are equivalent, however, the 9 hour decellularised sections exhibit a statistically significant decrease by comparison.

The non-decellularised and 9 hour non-decellularised sections exhibit no significant difference at any stage of the stress-strain response. After 15 hours decellularisation the same trends were exhibited in comparison to non-decellularised sections. However, there was no significant increase in the alteration in biomechanical properties found as a result of a prolonged treatment time when compared to 9 hour decellularised sections, see Figure 4.9 and Table 4.1.

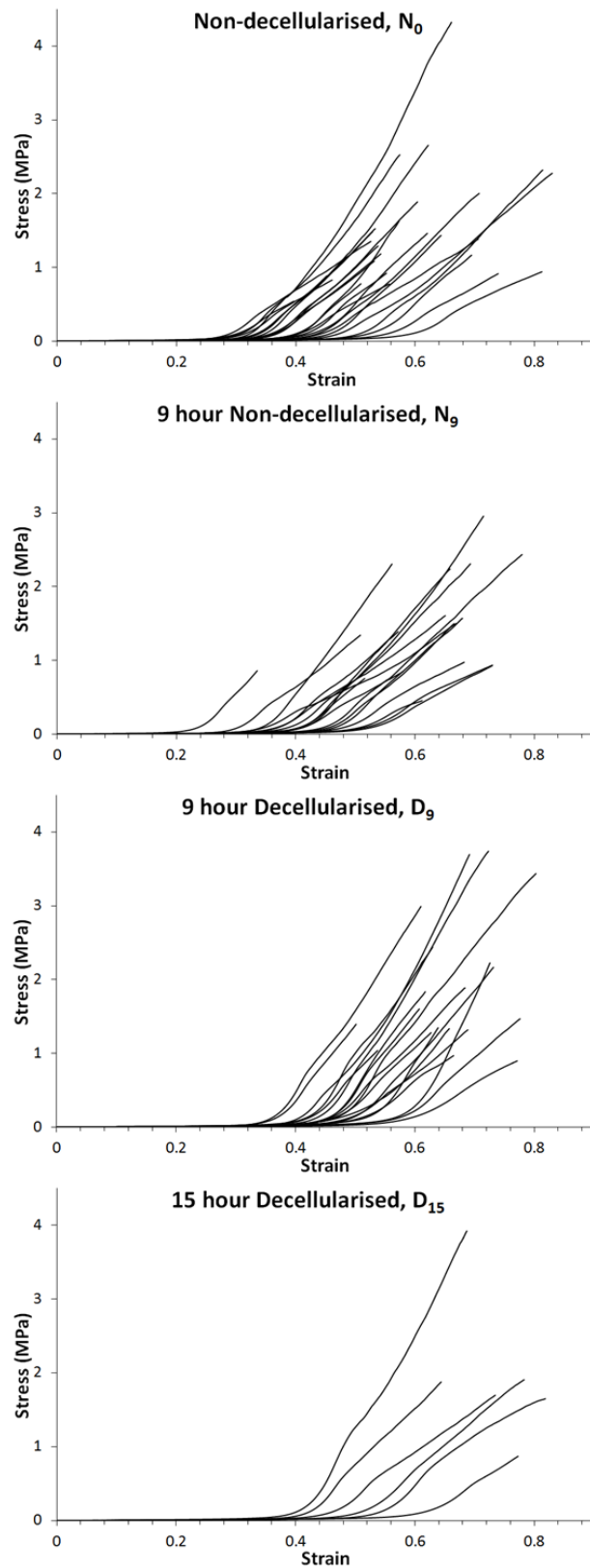


Figure 4.7: Uniaxial stress-strain response for porcine right coronary arteries; non-decellularised (N_0 , $n=25$), 9 hour non-decellularised (N_9 , $n=18$), 9 hour decellularised (D_9 , $n=19$) and 15 hour decellularised (D_{15} , $n=6$).

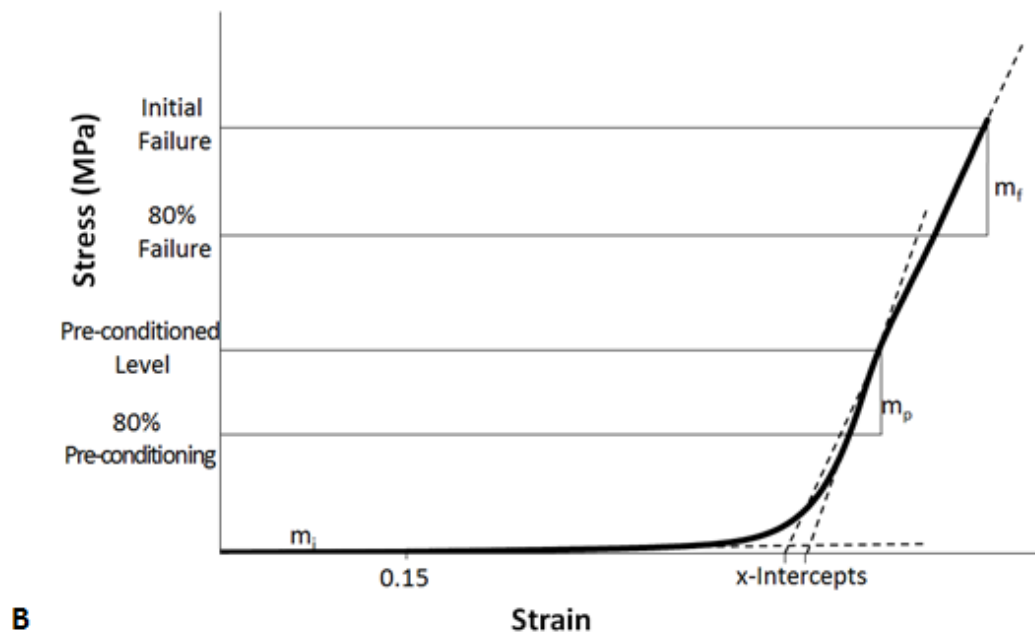
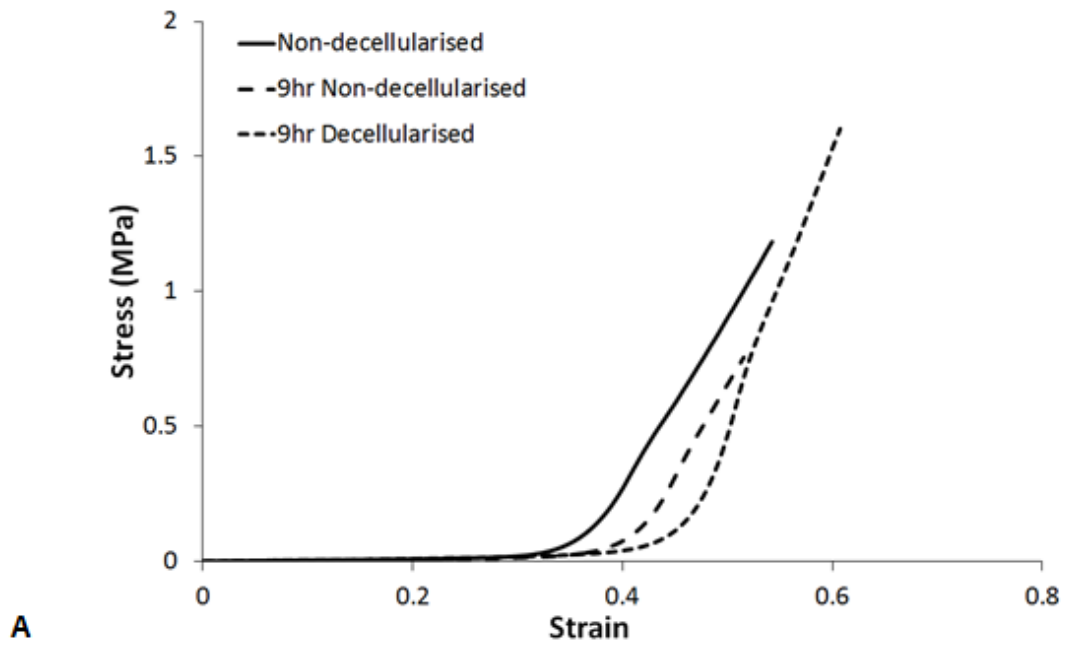


Figure 4.8: Uniaxial stress-strain response of porcine coronary arteries.
(A) Representative uniaxial stress-strain curves for non-decellularised, 9 hour non-decellularised and 9 hour decellularised right coronary artery sections.
(B) Determination of initial slope, m_i , pre-conditioned slope, m_p , final slope, m_f , and x-intercepts for m_p and m_f .

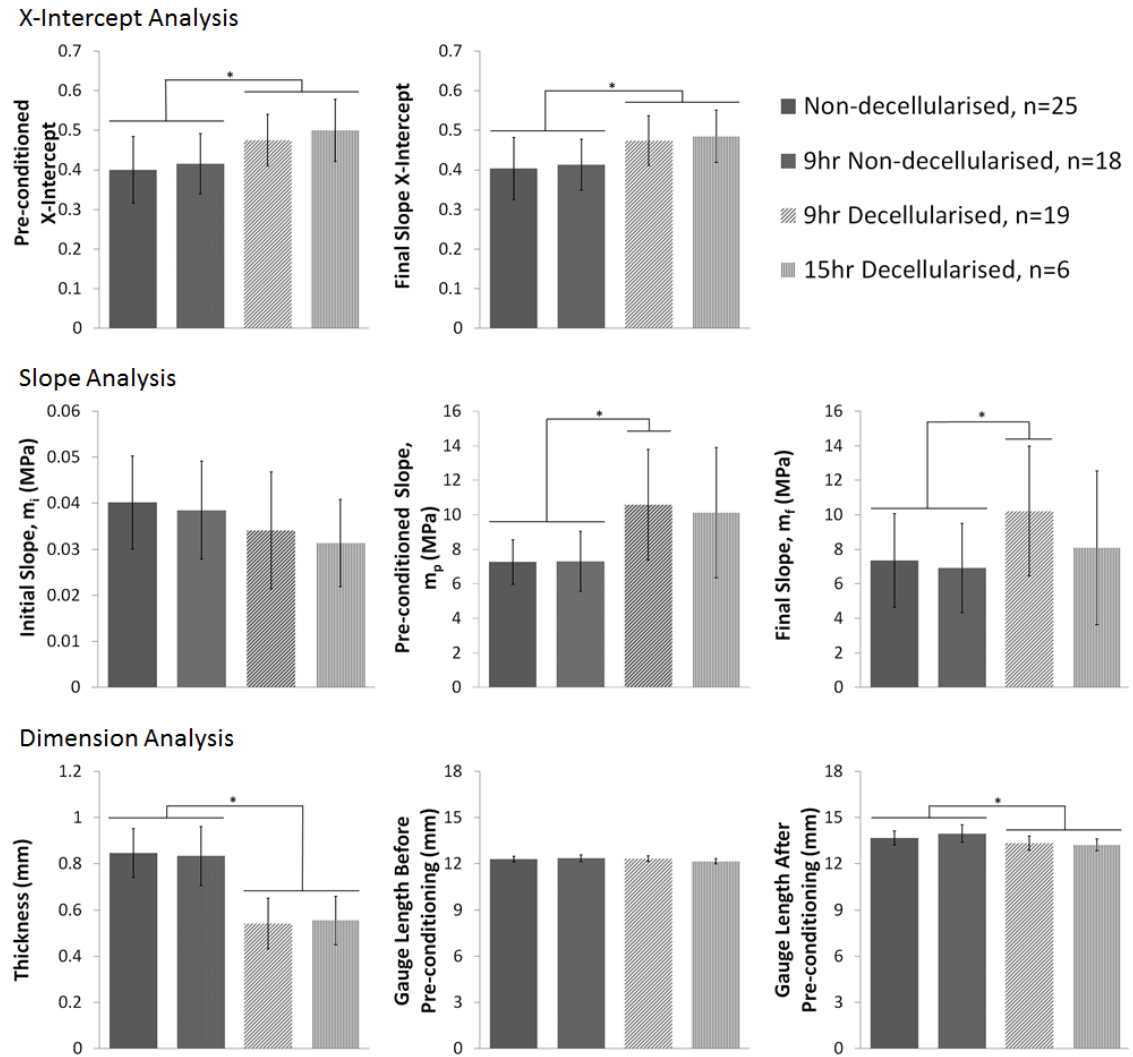


Figure 4.9: Uniaxial tensile data for non-decellularised, 9 hour non-decellularised, 9 hour decellularised and 15 hour decellularised sections of right coronary arteries. Significance, * $p < 0.05$.

Table 4.1: Uniaxial tensile data for non-decellularised (N_0), 9 hour non-decellularised (N_9), 9 hour decellularised (D_9) and 15 hour decellularised (D_{15}) sections of right coronary arteries as represented in Figure 4.9.

	Initial Slope, m_i (kPa)	Pre-conditioned Slope, m_p (MPa)	Final Slope, m_f (MPa)	Pre-conditioned X-Intercept	Final X-Intercept	Thickness (mm)	Length Before Pre-conditioning (mm)	Length After Pre-conditioning (mm)
$N_0, n=25$	40.2 ± 10.1	7.27 ± 1.29	7.35 ± 2.71	0.40 ± 0.08	0.40 ± 0.08	0.85 ± 0.12	12.30 ± 0.18	13.67 ± 0.46
$N_9, n=18$	38.5 ± 10.6	7.30 ± 1.73	6.91 ± 2.58	0.42 ± 0.08	0.41 ± 0.06	0.83 ± 0.13	12.36 ± 0.21	13.96 ± 0.56
$D_9, n=19$	34.1 ± 12.7	$10.58 \pm 3.21^{1,2}$	$10.21 \pm 3.77^{1,2}$	$0.48 \pm 0.07^{1,2}$	$0.47 \pm 0.06^{1,2}$	$0.54 \pm 0.11_{1,2}$	12.34 ± 0.18	$13.33 \pm 0.46^{1,2}$
$D_{15}, n=6$	31.3 ± 9.4	$10.13 \pm 3.77^{1,2}$	8.08 ± 4.45	$0.50 \pm 0.08^{1,2}$	$0.48 \pm 0.07^{1,2}$	$0.55 \pm 0.10^{1,2}$	12.16 ± 0.17	$13.23 \pm 0.39^{1,2}$
Significance, ¹ $p < 0.05$ from non-decellularised (N_0) sections								
² $p < 0.05$ from 9 hour non-decellularised (N_9) sections								

4.2.2 Inflation Tests

The pressure-external diameter and pressure-diametrical stretch curves for inflation tests were analysed as shown in Figure 4.10. A linear fit of the data resulted in measurements of the initial slope, m_i , the physiological slope between 80 mmHg and 120 mmHg, m_{phy} , and the final slope, m_f .

The external diameter-pressure curves for each LAD demonstrate that non-decellularised and decellularised arteries exhibit initial and final linear regions linked by a non-linear region, see Figure 4.11. The external diameter at the initial reading of 10 mmHg increased from 3.80 ± 0.47 mm to 4.33 ± 0.57 mm after 9 hour decellularisation however this was not statistically significant. 9 hour decellularised LADs experience a larger external diameter at all recorded pressures compared to non-decellularised LADs. Statistical significance was found in the slopes for 9 hour decellularised LADs in comparison to non-decellularised LADs at all stages of the external diameter-pressure curves, see Table 4.2.

The pressure-diametrical stretch curves show that 9 hour decellularised LADs are initially less stiff when compared to non-decellularised LADs, see Table 4.3 and Figure 4.12. However in all but one instance, as the pressure increased the 9 hour decellularised LADs exhibited stiffer behaviour than non-decellularised LADs.

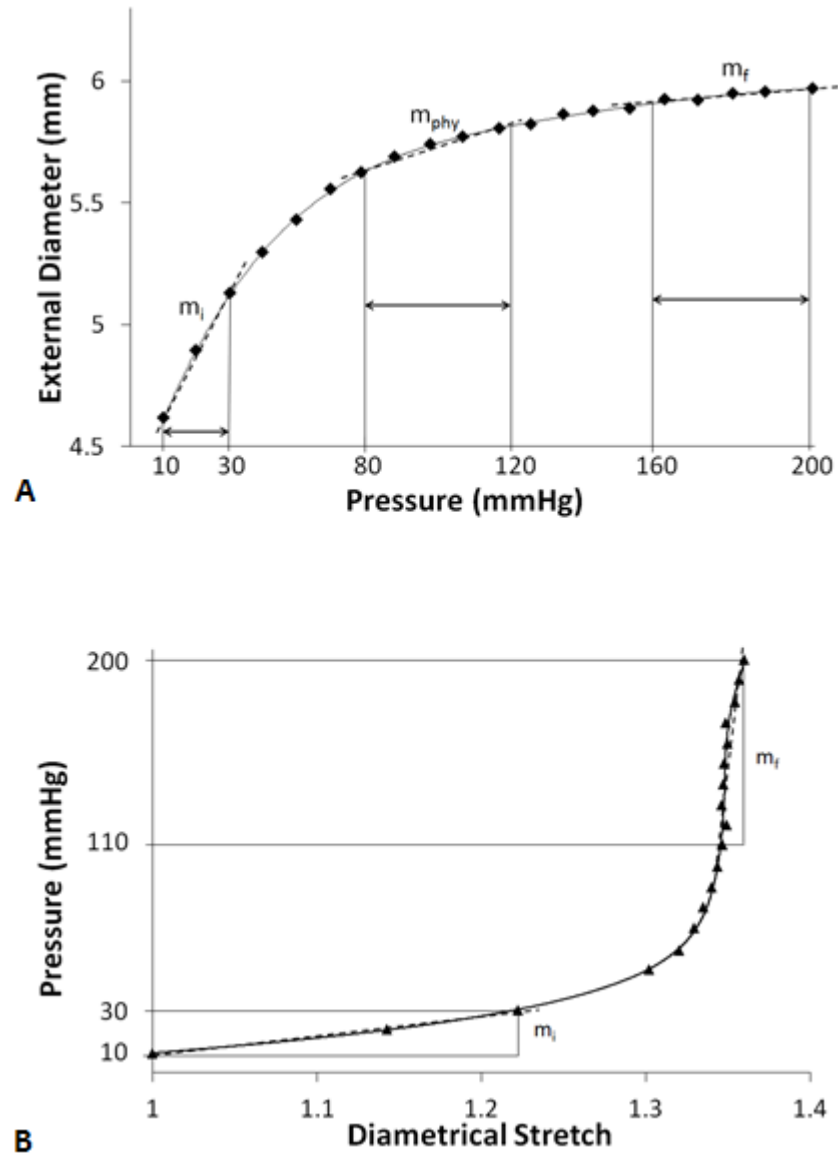


Figure 4.10: Analysis of inflation test data. (A) Determination of initial slope, m_i , physiological slope, m_{phy} , and final slope, m_f , of the pressure-external diameter response, and (B) determination of the initial (m_i) and final slopes (m_f) of the pressure-diametrical stretch response for inflation tests of porcine left anterior descending coronary arteries.

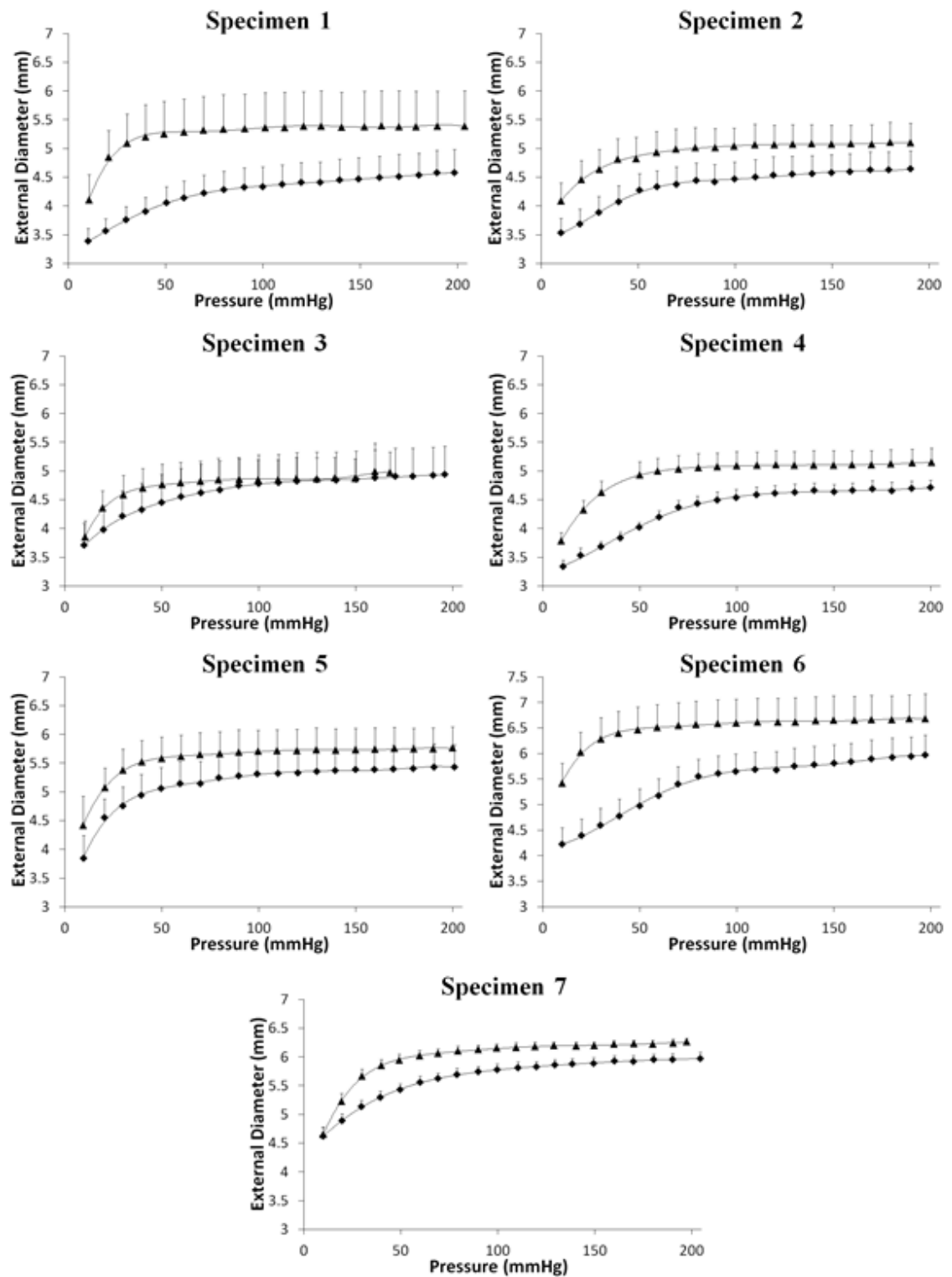


Figure 4.11: Pressure-external diameter response for inflation tests of non-decellularised (♦) and corresponding 9 hour decellularised (▲) left anterior descending porcine coronary arteries. Markers and error bars indicate mean + one standard deviation.

Table 4.2: Non-decellularised (N_0) and 9 hour decellularised (D_9) pressure-external diameter data analysis from inflation tests of porcine left anterior descending porcine coronary arteries.

	Initial Slope, m_i (mmHg)	Physiological Slope, m_{phy} (mm/mmHg)	Final Slope, m_f (mmHg)	Initial External Diameter (mm)
N_0 , n=7	0.0244 ± 0.0099	0.0032 ± 0.0006	0.0017 ± 0.0008	3.80 ± 0.47
D_9 , n=7	$0.0423 \pm 0.0083^*$	$0.0013 \pm 0.0007^*$	$0.0007 \pm 0.0003^*$	4.33 ± 0.57
Significance, * $p < 0.05$				

Table 4.3: Non-decellularised (N_0) and 9 hour decellularised (D_9) pressure-diametrical stretch data analysis from inflation tests of porcine left anterior descending porcine coronary arteries.

	Initial Slope, m_i (mmHg)	Final Slope, m_f (mmHg)
N_0 , n=7	167.49 ± 45.66	2087.99 ± 584.37
D_9 , n=7	$100.77 \pm 22.82^*$	$5769.38 \pm 1187.68^*$
Significance, * $p < 0.05$		

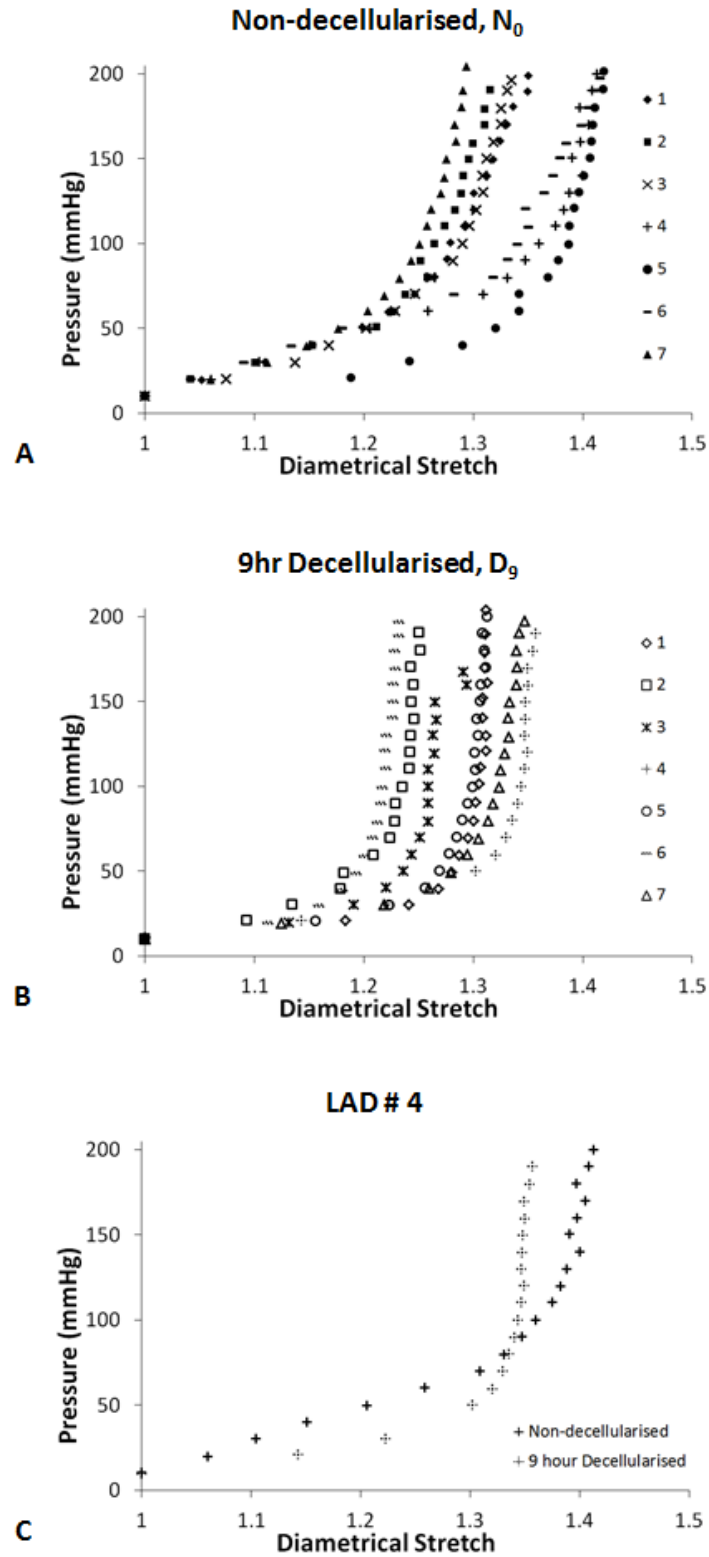


Figure 4.12: Pressure-diametrical stretch response for inflation tests of (A) non-decellularised and (B) corresponding 9 hour decellularised left anterior descending porcine coronary arteries. (C) Response of LAD # 4 before and after decellularisation.

4.2.3 Permeability Tests

A statistically significant increase in the permeability of 9 hour decellularised sections was exhibited in comparison to both non-decellularised and 9 hour non-decellularised sections, see Figure 4.13. The permeability increased from $3.5 \times 10^{-13} \text{ m}^2$ to $2.8 \times 10^{-12} \text{ m}^2$ following 9 hour decellularisation. There was no statistically significant difference in the permeability of non-decellularised LCX and 9 hour non-decellularised LCX. The permeability of the 9 hour decellularised sections displayed a large variability which was not present in the non-decellularised sections. All test groups withstood pressures up to 1,500 mmHg. The thickness of 9 hour decellularised sections of LCX, $0.43 \pm 0.10 \text{ mm}$, was a statistically significant reduction compared to non-decellularised and 9 hour non-decellularised sections, $0.74 \pm 0.14 \text{ mm}$ and $0.74 \pm 0.12 \text{ mm}$, respectively.

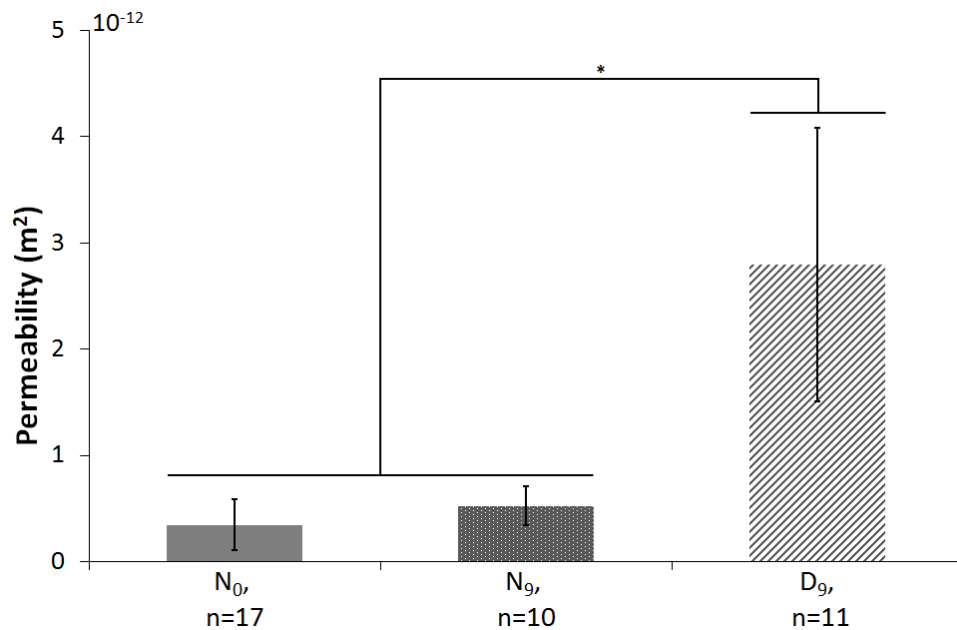


Figure 4.13: Permeability of non-decellularised (N_0), 9 hour non-decellularised (N_9) and 9 hour decellularised (D_9) sections of porcine left anterior descending coronary arteries. Significance, * $p < 0.05$.

4.2.4 Dimensional Analysis

Representative images of ring segments in the no-load and zero-stress state for non-decellularised and 9 hour decellularised PCAs are shown in Figure 4.14. Thickness, internal circumference, external circumference, and opening angle data are presented in Table 4.4. Upon opening, the non-decellularised section increased in thickness and internal circumference and decreased in external circumference although there is no statistical significance. 9 hour decellularised sections also exhibit a decrease in external circumference upon opening but to a lesser extent. However, there is no change in thickness or internal circumference. There is a statistically significant decrease in the thickness of 9 hour decellularised PCAs in comparison to non-decellularised sections for both no-load and zero-state configurations. The radial stretch ratio, calculated as $\lambda = T_n / T_z$, where T_n is the no-load wall thickness and T_z is the zero-stress wall thickness, was 0.91 for non-decellularised sections and 1.01 for 9 hour decellularised sections. The internal diameter of 9 hour decellularised PCA increased in comparison to non-decellularised PCA however no trend was found for the external circumference. The circumferential stretch ratio was calculated as $\lambda = C_n / C_z$, where C_n is the no-load

Table 4.4: Dimensional analysis of corresponding sections of non-decellularised (N_0) and 9 hour decellularised (D_9) porcine coronary arteries.

State		Thickness (mm)	Internal Circumference (mm)	External Circumference (mm)	Opening Angle (°)
N_0 , n=14	No-load	0.63 ± 0.18	6.24 ± 1.48	10.19 ± 2.06	-
	Zero-stress	0.70 ± 0.21	6.82 ± 1.34	9.01 ± 2.09	72.22 ± 26.42
D_9 , n=14	No-load	0.46 ± 0.11^1	7.20 ± 1.44	9.93 ± 1.72	-
	Zero-stress	0.46 ± 0.15^2	7.21 ± 1.51	9.63 ± 1.72	18.59 ± 27.52^2
Significance, ¹ $p < 0.05$, from corresponding no-load non-decellularised sections					
² $p < 0.05$, from corresponding zero-stress non-decellularised sections					

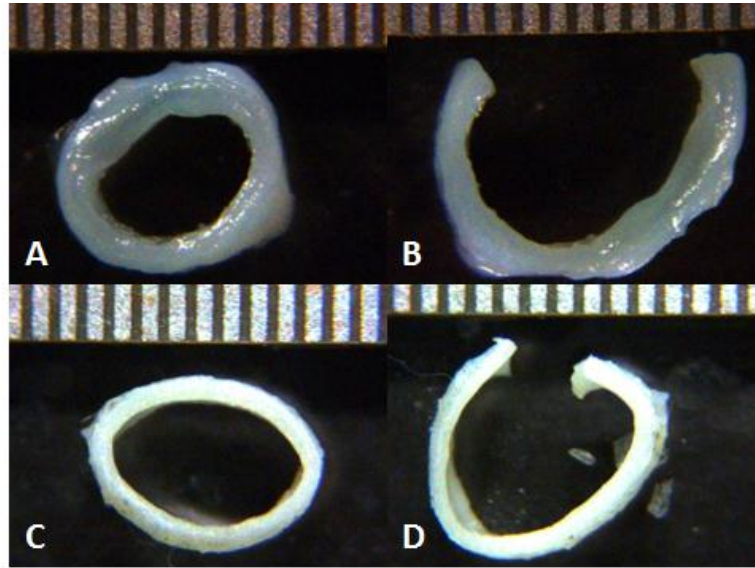


Figure 4.14: Dimensional analysis of (A, C) intact and (B, D) open sections of (A-B) non-decellularised and (C-D) 9 hour decellularised porcine coronary arteries.

circumference and C_z is the zero-stress circumference. The internal and external circumferential stretch ratios for non-decellularised sections were 0.91 and 1.13, respectively. The corresponding internal and external circumferential stretch ratios for 9 hour decellularised sections were 1 and 1.03, respectively. There was a statistically significant decrease in the opening angle for 9 hour decellularised PCAs compared to non-decellularised PCAs. The in situ longitudinal pre-stretch of ten LADs was measured as $28.9\% \pm 4.2\%$.

4.3 Discussion

There is limited data on the biomechanical properties of human and porcine coronary arteries in terms of uniaxial tensile, biaxial tensile and inflation tests [13,20,21]. This study characterises the biomechanical properties of small-diameter muscular porcine coronary arteries and is the first to provide an insight into the corresponding coronary artery natural matrix scaffold.

Due to their anisotropic nature, the non-linear response of arteries to an applied load is direction-dependent. In this study uniaxial tensile tests were performed in the

longitudinal direction only due to limitations relating to the dimensions of PCAs. However, this provides sufficient information on the stress-strain response of PCAs and the corresponding alteration due to decellularisation. Although statistically significant changes in the stress-strain response occur as a result of the short-term decellularisation method, the natural matrix scaffold maintains the highly non-linear response of non-decellularised arteries. This indicates a gross preservation of the collagen and elastin matrix. An initial linear region due to the extension of elastin fibres is followed by a highly non-linear region as collagen fibres straighten out and a final linear region once all the collagen fibres are recruited. Similar to other studies of decellularised arteries, the natural matrix scaffold of PCA is initially less stiff and more distensible in the low load range compared to non-decellularised sections [90,100], see Figure 4.9 and Table 4.1. Subsequently, as undulated collagen straightens out and is recruited, the decellularised arteries become stiffer than non-decellularised arteries [64,90,92]. The absence of smooth muscle cells results in a more pronounced response from the collagen and elastin content; an increased elasticity in the low-load range due to the elastin and an increase in stiffness in the latter stages from the contribution of collagen fibres. Uniaxial tensile tests performed after 15 hours decellularisation demonstrate that there is no further significant alteration in the biomechanical properties of the scaffold despite the increased porosity observed in Chapter 3. This verifies its ability to withstand loads despite the extended treatment time.

Inflation tests exhibit similar trends in the radial direction to longitudinal uniaxial tensile tests where the initially less stiff decellularised PCA becomes increasingly stiffer in comparison to the non-decellularised segments, see Figure 4.12 and Table 4.3, similar to the findings of Conklin et al. [8]. In contrast to other studies which test different vessels [8,36,65,104], the inflation tests were performed on the same LAD vessel before and after decellularisation, therefore, eliminating the variability associated with soft tissue testing. The initial compliant region of the pressure-external diameter curves where the diameter increases dramatically with increasing pressure is due to the contribution of elastin. A stiffer response is exhibited once the collagen fibres are recruited, see Figure 4.11. Decellularised PCAs reach a plateau after 60 mmHg as the collagen fibres are fully recruited and

resist further radial expansion. This is further demonstrated by the physiological and final slopes of decellularised arteries which were not significantly different. However, there is a statistically significant decrease in the final slope of non-decellularised arteries in comparison to the physiological slope indicating the continuing recruitment of collagen fibres and further expansion of the vessel. The physiological and final slopes of the 9 hour decellularised arteries are significantly less than that of non-decellularised arteries. Similar to other inflation studies, the initial diameter of decellularised arteries at 10 mmHg is larger than that of non-decellularised arteries [8,36,104]. With the exception of specimen 3, the external diameter of non-decellularised arteries does not expand to the same diameter as the corresponding decellularised vessel, see Figure 4.11.

Van Andel et al. [13] showed that young PCA is approximately three times more elastic than aged (61 to 85 years) human coronary arteries, see Figure 4.15. A circumferential strain of between 60% and 70% was achieved for porcine coronary arteries, however, a value of approximately 20% circumferential strain was achieved for human coronary arteries, see Figure 4.15 (B). The expansion of the external diameter of non-decellularised PCA in the range of 10 to 100 mmHg in this current study, see Figure 4.11, follows a similar trend to that seen by van Andel, see Figure 4.15 (A). However, van Andel shows the expansion of human coronary arteries from 0 to 100 mmHg is much less than that of PCAs. Additionally, circumferential stress in PCAs was lower at a given circumferential strain compared to human coronary arteries, see Figure 4.15 (B).

A key factor for vascular substitutes is burst pressure or ultimate strength since an inadequate burst pressure could lead to acute rupture and anastomotic degeneration [55]. In this current study, burst pressure tests have not been explicitly performed however inflation tests confirm that 9 hour decellularised tubular sections can withstand luminal pressures up to 200 mmHg. The permeability tests indicate that flat sections of 9 hour decellularised matrix can withstand pressures up to 1500 mmHg, well above the physiological range of an artery. Since this pressure only indicates the normal radial pressure that the tissue can withstand, without explicit consideration of circumferential stress, it can therefore not be used as a true measure of burst pressure but it does show that the tissue retains considerable

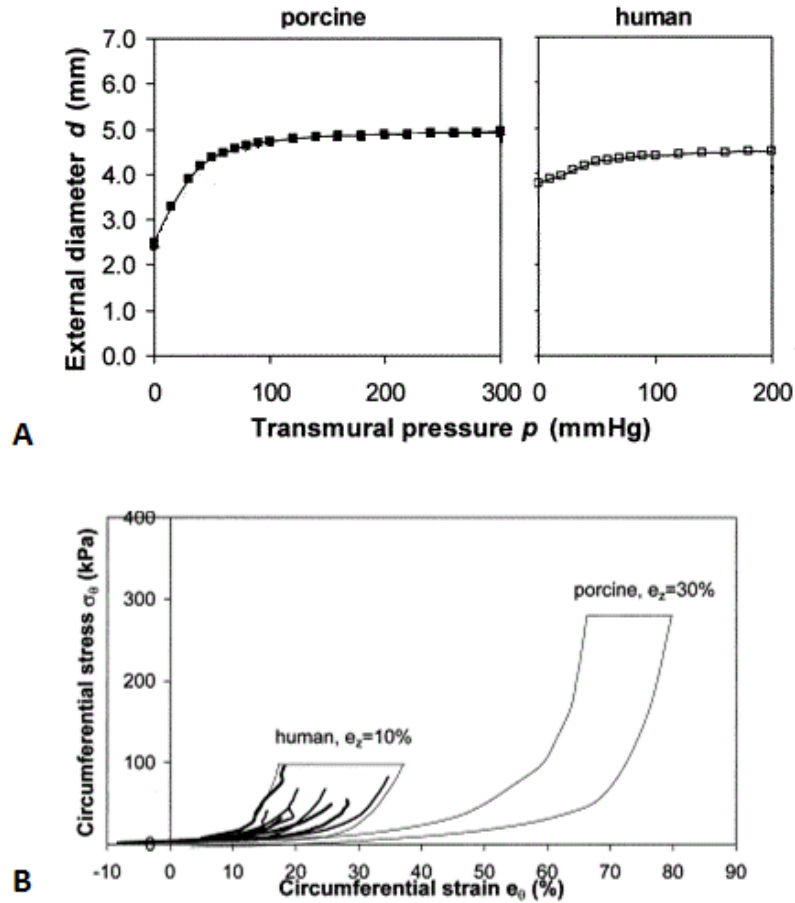


Figure 4.15: Comparison of inflation tests of porcine and human coronary arteries. (A) External diameter-transmural pressure curves and (B) circumferential stress-circumferential strain curves of young porcine and aged human left anterior descending coronary arteries (adapted from [13]).

integrity and strength following decellularisation. The burst pressure of coronary arteries has not been previously tested, however the burst pressures of human saphenous veins and internal mammary arteries are reported to be $1,599 \pm 877$ mmHg and $3,196 \pm 164$ mmHg respectively [66]. Other studies of decellularised vessels were unable to maintain pressures up to 200 mmHg [29,104] and, therefore, would be unsuitable for implantation as a vascular graft. Williams et al. [29] was unable to perform inflation tests on decellularised sections of rabbit carotid artery as the tissue was too porous following decellularisation and could not maintain a steady pressure. Decellularised porcine carotid arteries tested by Heine et al. [104] failed between pressures of 120 and 200 mmHg.

Additionally, permeability tests show flat sections of decellularised PCA can withstand pressures of up to 1,500 mm Hg. Permeability tests have not previously been performed on non-decellularised blood vessels. The permeability of human umbilical vein (HUV) has been measured over the course of a perfusion decellularisation procedure [108]. The transmural flow across a segment of HUV, 85 mm in length, was measured over 72 hours resulting in an average permeability of $2.3 \times 10^{-16} \text{ m}^2$ at 150 mmHg. This is considerably lower in comparison to the non-decellularised and decellularised values in this current study, see Figure 4.13. A significant increase in permeability of the tissue results following 9 hour decellularisation. The removal of material from the vascular wall can be linked to this 8-fold increase in permeability. This provides key information on the permeability of the natural matrix scaffold and therefore the optimum environment for vascular smooth muscle cells seeded in tissue engineered scaffolds. Additionally, the significant increase in permeability may encourage cell infiltration aiding in the repopulation of the scaffold.

Residual stresses are present in non-decellularised arteries in the no-load state, demonstrated by the springing open of the artery when a ring section is cut in the longitudinal direction, releasing the internal stresses. This implies that the internal surface, the intima, is undergoing compression and the outer surface, the adventitia, is undergoing tension in the no-load state [16]. The opening angle has been reported to significantly decrease as a result of the removal of vascular smooth muscle cells from the arterial wall [29,36]. Williams et al. [29] found a decrease from an average of 107° to 67° in the opening angles of non-decellularised and enzymatically decellularised rabbit common carotid arteries. Histological analysis confirmed that the elastic laminae remained intact indicating that increased porosity and disrupted cell-fibre interactions led to the relaxation in residual stresses. A reduction in opening angle from 69° for non-decellularised porcine carotid arteries to 26° for detergent-enzymatically treated vessels was found by Roy et al. [36]. They determined this was due to the release of compressive residual stress existing in the elastin fibre network as a result of the removal of vascular smooth muscle cells. In this current study, the opening angle of non-decellularised PCAs decreased from an average of 72° to 19° after decellularisation. The non-decellularised opening angle is

similar to that of Carboni et al. [35] who reported the opening angle of porcine left circumflex coronary artery as $75^{\circ} \pm 29^{\circ}$. The loss of residual stresses in the arterial wall can also account for the increase in initial external diameter of decellularised vessels compared to non-decellularised vessels in inflation tests, see Figure 4.11 and Table 4.2. Holzapfel et al. [127] demonstrated the residual stretches in the individual layers of human aorta in both the circumferential and longitudinal directions. When cut open to release residual stresses, the adventitia and media shortened while the intima elongated. Consequently, in this current study, the external circumference decreased and internal circumference increased in non-decellularised sections when opened, see Table 4.4. However, there was almost no change in these measured values for non-decellularised sections, further indicating the loss of residual stresses as a result of decellularisation.

Intuitively, the thickness of a blood vessel should decrease as a result of the removal of vascular cells. Similar to other decellularisation studies [36,65,114], decellularised PCAs experienced a decrease in wall thickness in comparison to non-decellularised PCAs, see Table 4.4. This is further supported by the thickness measurements of the tensile and permeability tests, see Figure 4.9 and Section 4.2.3.

The pre-stretch of human coronary arteries has been reported as 4.4% by Holzapfel et al. [127] and 10% by van Andel et al. [13]. In this current study, a standard pre-stretch of 30% was applied to LADs for inflation tests based on previous reports on the longitudinal stretch of porcine coronary arteries [13,36]. The use of this pre-stretch was validated by an average measurement of 29% pre-stretch PCAs in this study. Measuring and applying the unique pre-stretch of each individual artery may decrease the variability observed between arteries. Additionally, Roy et al. [36] reported a mean increase of 12% in the length of the elastic porcine carotid artery following detergent/enzymatic decellularisation. This was not accounted for in this study so further work could be completed to determine if there is any increase in length following 0.1M NaOH decellularisation.

The alteration in the biomechanical properties of non-decellularised and decellularised sections of PCA presented is due to the effects of the decellularisation method rather than a natural degradation of the tissue. This is verified by the comparison of the non-decellularised and 9 hour non-decellularised uniaxial tensile

test data and the permeability data where there is no statistically significant difference, see Figure 4.9 and Figure 4.13.

The biomechanical tests performed in this study provide an insight into the alteration of biomechanical properties of the PCA as a result of short-term decellularisation. Although changes in the biomechanical properties occur, the scaffold maintains the highly non-linear response present in non-decellularised arteries. Uniaxial tensile and inflation tests confirmed decellularisation resulted in a less stiff, more distensible low-load response and stiffer high-load response compared to non-decellularised sections. Additionally, extending the decellularisation treatment time yielded no significant increase in the changes already observed in the 9 hour decellularised sections. Chapter 5 will determine whether the increased permeability due to the removal of vascular cells is favourable for repopulation of the natural matrix scaffold. The natural matrix scaffold exhibited encouraging behaviour to perform as graft, withstanding pressures of 200 mmHg. Furthermore, the natural matrix scaffold obtained should also provide a suitable benchmark for the biomechanical properties of other scaffolds suitable for use as TEBV scaffolds.

Chapter 5 Repopulation Study and Biomechanical Evaluation of Decellularised Porcine Coronary Arteries

The previous chapters have demonstrated a short-term decellularisation method to achieve the natural matrix scaffold of porcine coronary arteries (PCA), maintaining the biomechanical and structural integrity of the tissue. However, for the PCA scaffold to perform as a tissue engineered blood vessel (TEBV), the ability to support xenogeneic vascular cell growth and withstand physiological pulsatile flow is vital. Vascular smooth muscle cells (vSMCs) are required to repopulate the medial layer of the natural matrix scaffold for extracellular matrix (ECM) production and smooth muscle tone. Endothelial cells (ECs) are necessary to form a confluent layer on the luminal surface of the scaffold to act as a smooth, non-thrombogenic lining.

To demonstrate the ability of the short-term decellularised PCA to maintain viable cells, the majority of tests were completed under static conditions using bovine smooth muscle cells, thereby confirming the ability of the scaffold to maintain xenogeneic cells. To determine the capability of cells to form a confluent layer on the abluminal surface, bovine aortic endothelial cells were seeded on the internal elastic lamina (IEL). Mesenchymal stem cells (MSCs) have demonstrated an ability to differentiate into vascular cells and are, therefore, a potential source of autologous cells. Consequently, in a preliminary study, MSCs were seeded to the PCA natural

matrix scaffold to assess the suitability for stem cell growth. Additionally, to validate PCA natural matrix scaffold for human applications an additional seeding test using human coronary artery smooth muscle cells was performed. To determine its ability to perform as a TEBV tubular lengths of the PCA scaffold were maintained in a bioreactor under pulsatile flow at physiological pressures. There is limited research completed to-date on the effect of cell seeding of decellularised scaffolds on the biomechanical properties. Therefore, to establish the influence of cell attachment and infiltration a biomechanical analysis of the seeded scaffold was completed using uniaxial tensile tests.

To investigate the potential of the natural matrix scaffold of PCA to act as a vascular graft or scaffold the following criteria were assessed:

- The ability of the PCA natural matrix scaffold as a suitable environment for xenogeneic cell attachment;
- The most appropriate surface of the scaffold to achieve cell infiltration;
- The maintenance of internal elastic lamina on the luminal surface of the scaffold as a barrier to cell infiltration;
- The capability of endothelial cells to adhere to and form a smooth, confluent lining on the luminal surface of the scaffold;
- The effect of cell infiltration on the biomechanical properties of the scaffold;
- The effect of a dynamic uniaxial strain environment on the growth of cells seeded to the scaffold;
- The viability of the scaffold to act as a graft by maintaining physiological pressure in the long-term;
- The ability of the scaffold to support human coronary artery smooth muscle cells.

5.1 Materials and Methods

All cell culture techniques were performed under sterile conditions in a Bio Air 2000 MAC laminar flow cabinet using sterile equipment. Cells were visualized using an Olympus CK30 phase contrast microscope. Cells and cell-seeded scaffolds were

maintained in a Nuair CO₂ water-jacketed incubator in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C.

5.1.1 Cell Culture

Cells were cultured in 25 cm², 75 cm², and 175 cm² tissue culture flasks under normal conditions and were routinely fed every 2 - 4 days. Cells were sub-cultured at 80 - 90% confluency by trypsinisation.

5.1.1.1 Bovine Aortic Smooth Muscle Cell and Bovine Aortic Endothelial Cell Culture

The main cell line used in this study was bovine aortic smooth muscle cells (BASMC, AG08504, Coriell, NJ, USA). Experiments were conducted between passages 8 - 18. Additionally, bovine aortic endothelial cells (BAEC, AG08500, Coriell, NJ, USA) were obtained and experiments were conducted between passages 14 - 18.

BASMC and BAEC were maintained in RPMI-1640 media (R8758, Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, F9665, Sigma-Aldrich) and 1% Penicillin-Streptomycin (P/S, P4333, Sigma-Aldrich) under normal conditions. To achieve sub-culture, the cells were washed twice in 1x phosphate-buffered saline (PBS, P5493, Sigma-Aldrich). Cells were removed from the bottom of the flask by incubating at 37 °C for 5 minutes in 2x Trypsin-EDTA (T4174, Sigma-Aldrich) diluted from a 10x stock solution. RPMI-1640 medium containing 10% FBS and 1% P/S was added to deactivate the trypsinisation process and the cell suspension was centrifuged at 1,500 RPM for 5 minutes to form a pellet. Cells were resuspended in culture medium and typically diluted 1:4 into culture flasks.

5.1.1.2 Rat Mesenchymal Stem Cell Culture

Rat mesenchymal stem cells (rMSCs, 009, REMEDI, Galway) were cultured between passages 7 - 14 under normal conditions. Cells were maintained in 50:50 Minimum Essential Medium Eagle Alpha Modified (α -MEM, M0644, Sigma-Aldrich) and Ham's Nutrient Mixture F12 (Ham's, 51651C, Sigma-Aldrich)

supplemented with 10% FBS and 1% P/S under normal conditions. Sub-culturing was performed as described in Section 5.1.1.1, using a concentration of 0.25x Trypsin-EDTA solution and appropriate media.

5.1.1.3 Human Coronary Artery Smooth Muscle Cell Culture

Human coronary artery smooth muscle cells (HCASMC, CC-2583, Lonza, Switzerland) were cultured between passages 2 - 5. HCASMC were maintained in smooth muscle cell growth medium 2 and Supplement Mix (C-22062, PromoCell) supplemented with 10% FBS and 1% P/S under normal conditions. The supplement mix contained 0.05 ml/ml fetal calf serum, 0.5 ng/ml epidermal growth factor, 2 ng/ml basic fibroblast growth factor, and 5 µg/ml insulin. Sub-culturing was performed as described in Section 5.1.1.1, using a concentration of 0.25x Trypsin-EDTA solution and appropriate media.

5.1.2 Cryogenic Cell Storage and Recovery

For long-term cell storage, cells were maintained in liquid nitrogen in a cryofreezer unit. Briefly, cells were washed in 1x PBS, trypsinised for 5 minutes at 37 °C, centrifuged at 1,500 RPM for 5 minutes to form a pellet, and re-suspended in cell freezing media containing 10% dimethylsulphoxide (cell freezing media-DMSO, C6164, Sigma-Aldrich). 1 ml aliquots were transferred to cryovials and frozen to -80 °C at a rate of 1 °C/minute in a Nalgene cryofreezing container. The cryovials were then transferred to liquid nitrogen.

To recover cells from long-term storage cryovials were rapidly thawed by gentle agitation in a waterbath at 37 °C. The cells were re-suspended in fresh growth medium in a 75 cm² tissue culture flask. The following day the cells were washed in PBS and fresh growth medium was added.

5.1.3 Tissue Preparation

PCA were excised from hearts as described in Section 3.1.1 (pg. 44). PCA were decellularised by perfusion for 9, 12, and 15 hours. Non-decellularised PCA were stored in 0.9% saline at room temperature for use as controls. To sterilize the tissue

suitable for cell culture, it was thoroughly washed in sterile PBS and placed in 70% ethanol (EtOH) for 1 hour in a laminar flow cabinet. The tissue was then washed three times in sterile PBS and placed in fresh PBS for 1 hour to rehydrate prior to cell seeding.

All equipment for cell seeding tests was obtained in pre-sterilised packaging or autoclaved prior to introduction into the laminar flow cabinet. No adhesion molecules were used in any experiment.

5.1.4 Static Cell Seeding of Scaffold

For static experiments, ligated branches were removed from tubular segments of PCA. The segments were opened in the longitudinal direction, divided into 5 x 5 mm sections, and placed in individual wells of culture plates using sterile tweezers. Sections containing branches were discarded. Sections of non-decellularised PCA and non-seeded decellularised PCA were maintained as controls and to determine the natural degradation of PCA and PCA scaffold with time. Seeded scaffolds and controls were maintained in an incubator under normal conditions for the duration of experiments.

5.1.4.1 Cell Attachment Study

To evaluate the suitability of decellularised PCA for cell attachment, BASMC were seeded to the luminal or abluminal sides of 5 x 5 mm sections of 9 hour decellularised PCA in a 24-well plate. BASMC were seeded at a concentration of 1×10^5 cells/cm². The cell-seeded scaffolds were maintained in RPMI-1640 media supplemented with 10% FBS and 0.2% Primocin (ant-pm-1, CAYLA-InVivoGen, France). To determine cell attachment after 24 hours, the luminal and abluminal surfaces were analysed by DAPI staining. Further sections were maintained for 72 hours and were processed for H&E staining to observe cell attachment to the abluminal and luminal surfaces.

5.1.4.2 Cell Infiltration Study

To determine the capability of cells to infiltrate PCA scaffolds, PCAs were decellularised for 9, 12, and 15 hours. 5 x 5 mm sections, abluminal surface facing upwards, were placed in 24-well plates using sterile tweezers. BASMC were seeded at a concentration of 5×10^4 cells/cm² in RPMI-1640 media supplemented with 10% FBS and 0.2% Primocin. Sections were removed from the experiment after 5, 10, 15, 30, and 45 days and processed for histological and immunocytochemical analysis.

To encourage the infiltration of cells into the scaffold, tubular segments of 15 hour decellularised PCA were perfused and soaked in 100% FBS for 1 hour, respectively. Additionally, the effect of increasing the cell seeding density was analysed. Eighteen 5 x 5 mm sections, abluminal side up, were placed in 24-well plates. BASMC were seeded at a concentration of 5×10^4 cells/cm² to 9 sections and at a concentration of 2×10^5 cells/cm² to a further 9 sections, see Figure 5.1. Three sections for each cell concentration were removed after 5, 10, and 15 days and processed for histological and immunocytochemical analysis.

		Day 5	Day 10	Day 15
Seeding Concentration	5×10^4 cell/cm ²	X	X	X
		X	X	X
		X	X	X
	2×10^5 cell/cm ²	X	X	X
		X	X	X
		X	X	X

Figure 5.1: Experimental set-up for BASMC-seeding of 15 hour decellularised PCA at high (2×10^5 cells/cm²) and low (5×10^4 cells/cm²) cell-seeding concentrations for periods of 5, 10, and 15 days. x: 5 x 5 mm section of decellularised PCA, abluminal side up.

5.1.4.3 Assessment of Endothelial Layer Formation

BAEC were seeded to the luminal surface of six 5 x 5 mm sections of 15 hour decellularised PCA at a concentration of 2×10^4 cells/cm² and incubated in RPMI-1640 media supplemented with 10% FBS and 0.2% Primocin. Three sections were removed after 24 hours and 10 days of culture, respectively, and prepared for histology and immunocytochemical analysis.

5.1.5 Cell Seeding of Tubular Scaffold

To show attachment by circulating cells, a 40 mm section of 15 hour decellularised PCA was placed in a test tube with BASMC-containing RPMI-1640 media supplemented with 10% FBS, 0.2% Primocin and 1.15g/500ml 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, H4034, Sigma-Aldrich). The BASMC concentration was 2×10^4 cells/cm². The test tube was attached to a rotator (Rotator Drive STR4, Stuart Scientific) which revolved at a speed of 1 RPM in an incubator under normal conditions. The media was replaced with fresh BASMC-containing media (concentration of 2×10^4 cells/cm²) every 24 hours for 7 days. After 8 days the section was removed, washed in sterile PBS, divided into seven equal sections, and prepared for histological and immunocytochemical analysis.

5.1.6 Effect of Uniaxial Cyclic Strain on BASMC-seeded Scaffold

The FX-4000TM Flexercell® Tension PlusTM system (Flexcell International Corp, McKeesport, PA, USA) consists of an electronic control unit, a vacuum pump, and a baseplate containing BioFlex® 6-well plates (Dunn Labortechnik GmbH, Asbach, Germany), see Figure 5.2 (A). A vacuum deforms the compliant silicone membranes of the BioFlex® plates over the loading posts of the baseplate, see Figure 5.2 (C), thereby applying a controlled strain to cells seeded to the membrane. A method was developed to attach PCA sections to the silicone membrane of the BioFlex® 6-well plates. 15 x 5 mm sections of PCA were obtained using a custom-made die. PCA sections were sutured to 15 x 5 mm sections of silicone using sterile needle and thread (Johnson and Johnson, Mersilk W622H) with the aid of a custom-made die for uniformity, see Figure 5.3. The die consists of three plates which slot together. The

middle plate has an opening for two silicone sections and one section of PCA. The top and bottom plates have guides for the needle and thread. The silicone sections, with PCA attached, were then located in a well of the BioFlex® plate using a custom die, see Figure 5.4. The silicone sections were glued (Momentive Silicone Rubber Adhesive Sealant) to the silicone membrane of the plate. 200 µL of PBS was pipetted onto PCA to maintain hydration for 1 hour while the glue dried. A mean strain of 5%, with an amplitude of $\pm 2.5\%$ and at a frequency of 1 Hz, was achieved by calibration using a video extensometer. This loading regime was chosen to represent the approximate strains induced in an artery under physiological conditions [128]. The sutures on the PCA scaffold were used as markers to calibrate the system.

Eight sections, 15 x 5 mm, of 9 hour decellularised PCA were attached to two BioFlex® plates, luminal surface facing upwards. Following calibration, two scaffolds on each plate were seeded with BASMC at a concentration of $1.25 \times 10^5 \text{ cm}^{-2}$ in RPMI-1640 media supplemented with 10% FBS and 0.2% Primocin. The BioFlex® plates were incubated under normal conditions for 24 hours to allow for cell attachment. Strain was applied to one plate for a further 72 hours whilst the other plate remained unstrained as a control. The sections were then removed from the silicone membranes and processed for immunocytochemical analysis.

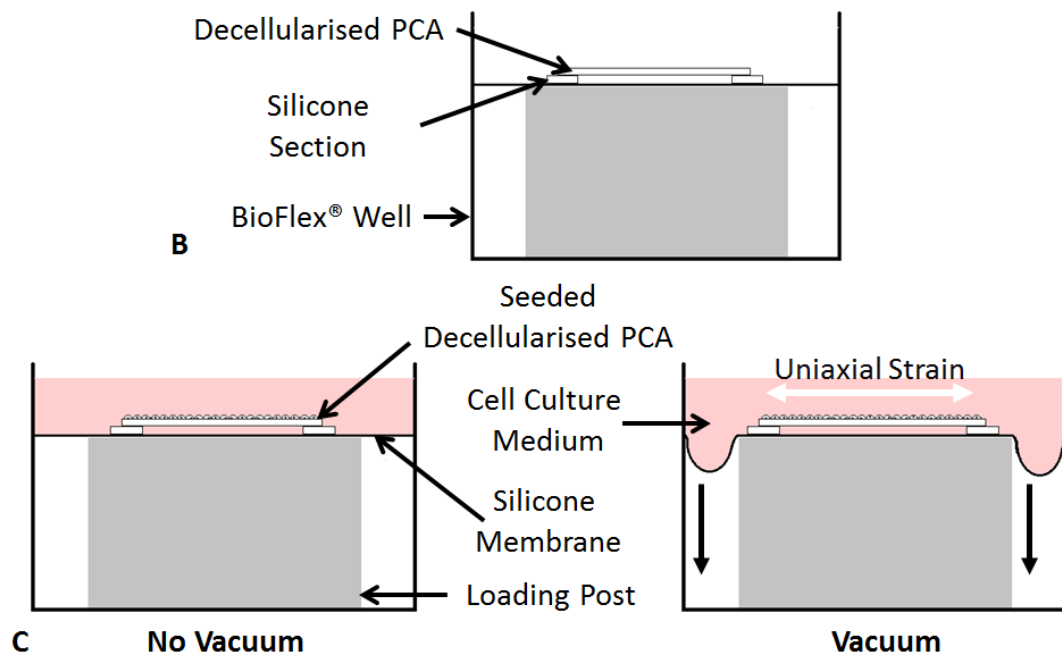
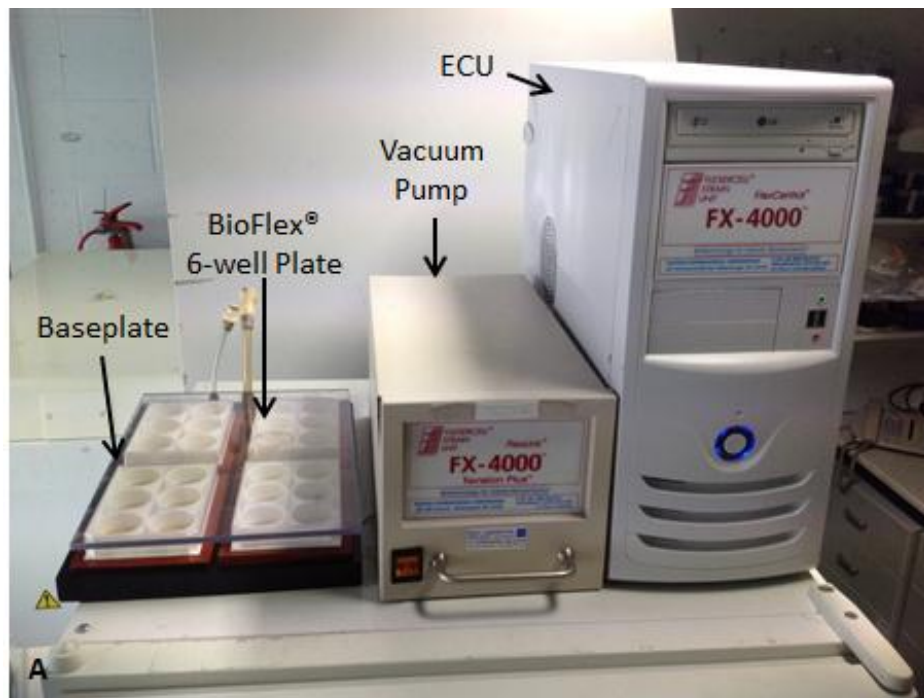


Figure 5.2: Application of uniaxial strain to porcine coronary artery section. (A) FX-4000TM Flexercell® Tension PlusTM system; (B) schematic of porcine coronary artery section attached to the silicone membrane of a BioFlex® Well via silicone sections glued to the surface; and (C) application of uniaxial strain to BASMC-seeded scaffold.

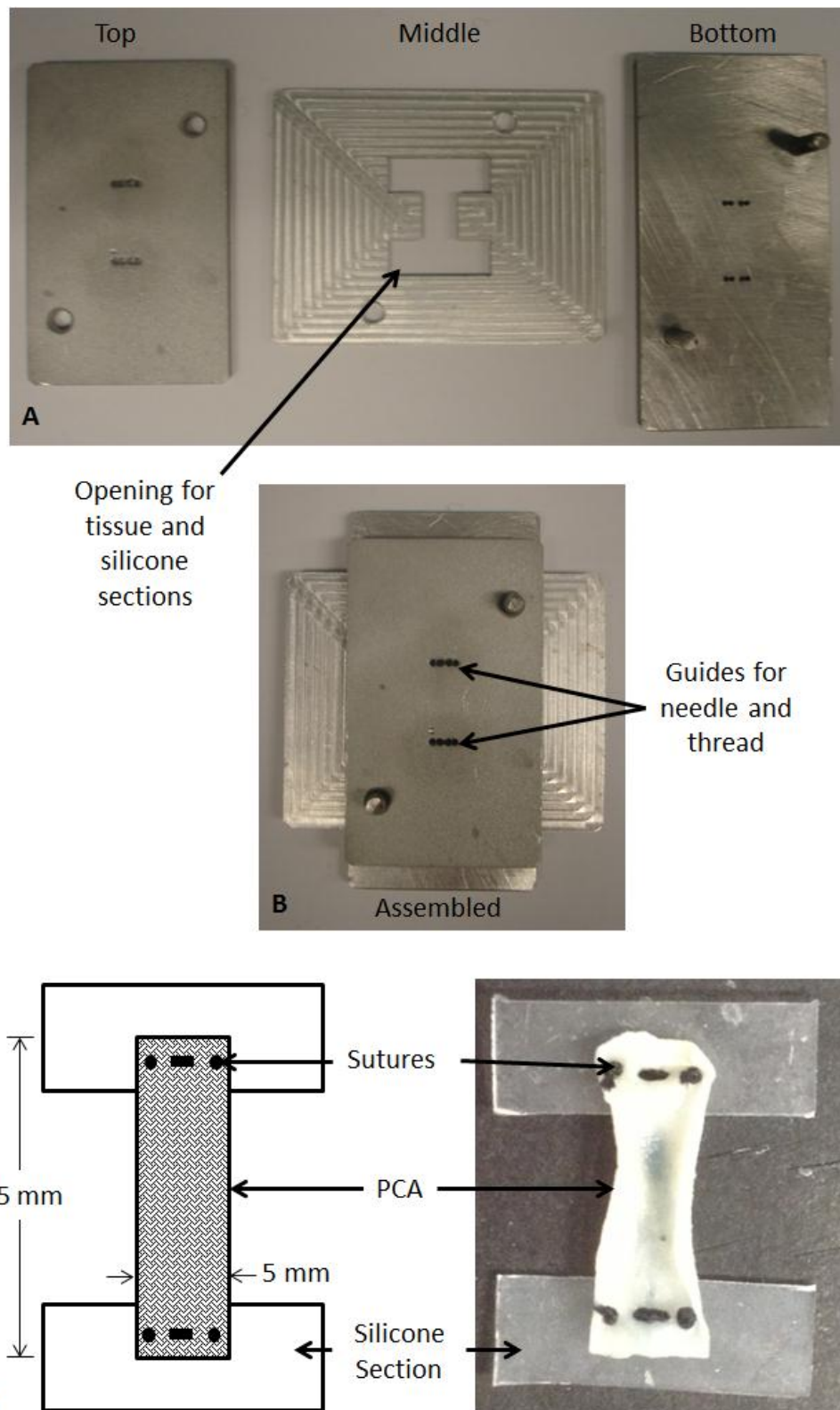


Figure 5.3: Attachment of porcine coronary artery sections to silicone sections. (A-B) Custom die to uniformly suture tissue to silicone sections; and (C) completed section.

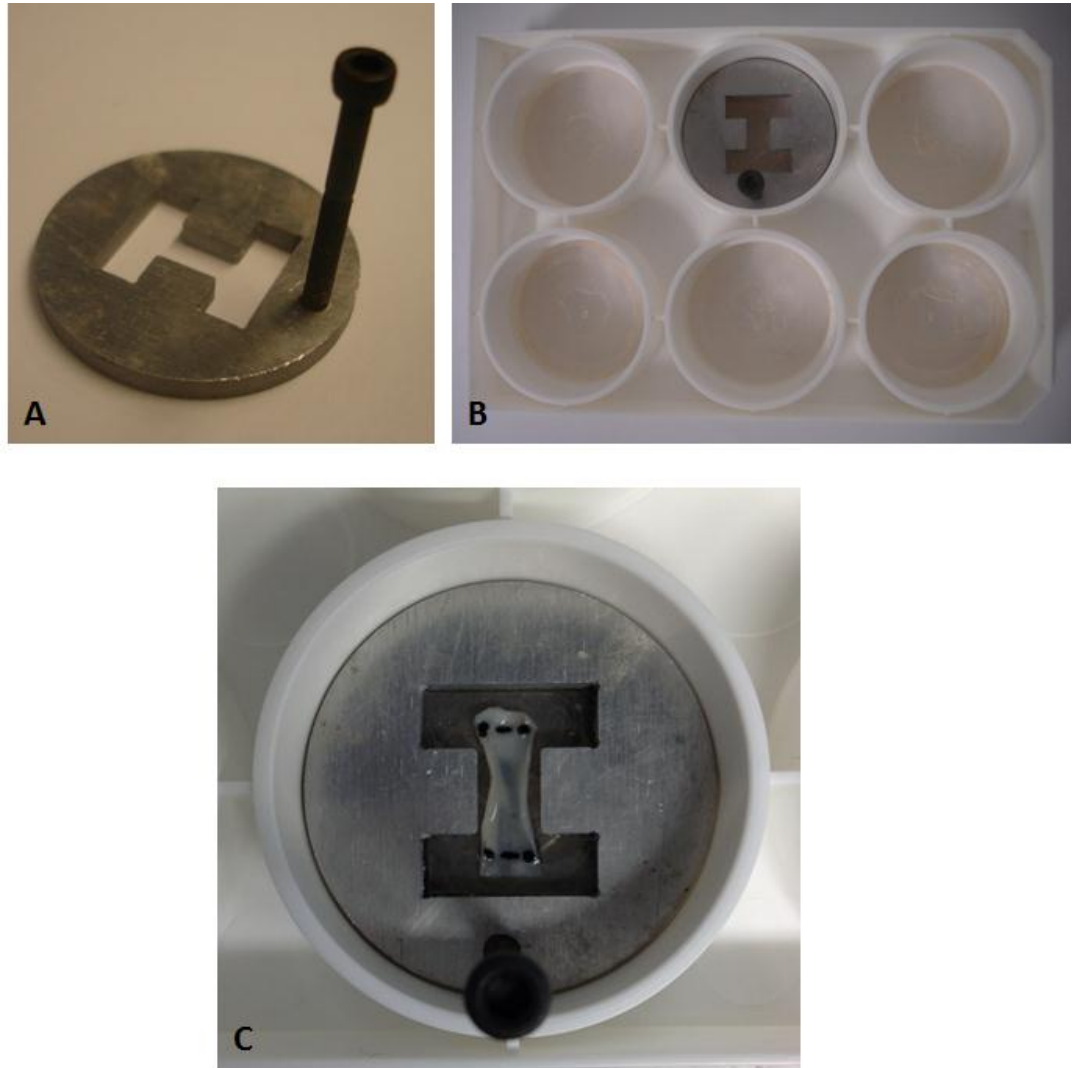


Figure 5.4: Attachment of porcine coronary artery sections to BioFlex® Well.
(A) Custom die for uniform placement of sections; (B-C) alignment of die and sutured section in BioFlex® Well.

5.1.7 Biomechanical Analysis of BASMC-seeded Scaffold

Nine PCAs were obtained to determine of the effect of cell seeding and infiltration on the biomechanical properties of PCA scaffolds. Six PCAs were decellularised for 15 hours and three were maintained in 0.9% saline as non-decellularised controls. Where possible, lengths of PCA were divided into two consecutive sections, see Figure 5.5, resulting in ten 15 x 5 mm decellularised sections and three 15 x 5 mm non-decellularised sections. All sections were placed in 24-well plates, abluminal surface facing upwards, and incubated in RPMI-1640 media supplemented with 10% FBS and 0.2% Primocin under normal conditions. Five decellularised sections were seeded with BASMC at a concentration of 2×10^4 cells/cm² every day for 7 days. All sections were removed after 30 days. Uniaxial tensile tests were performed using a similar protocol to Section 4.1.2 (pg. 69). Each section was divided in the longitudinal direction, each 15 x 2.5 mm, see Figure 5.5. Pre-conditioning was performed to 0.5 N over 5 cycles and sections were then tested to failure. Sections were fixed in 10% formalin for histological analysis.

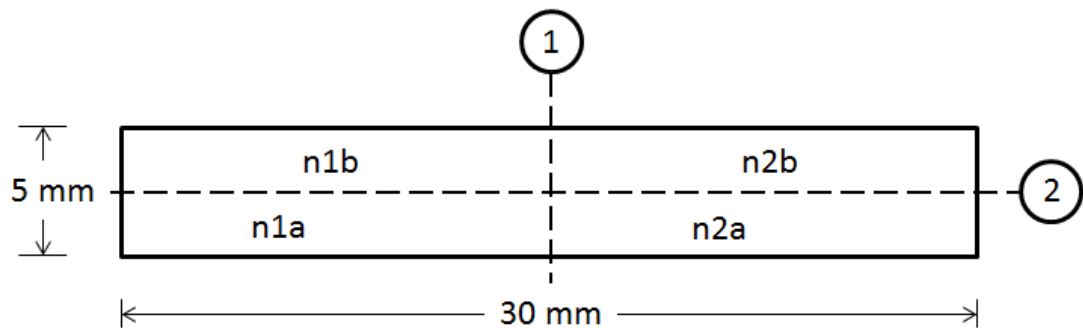


Figure 5.5: Division of porcine coronary artery for uniaxial tensile tests. Cut 1 was made prior to placement in 24-well plates, dividing the artery into two consecutive sections. Cut 2 was made upon completion of the experiment, enabling uniaxial tensile tests of up to 4 sections from the same artery.

5.1.8 Effect of Pulsatile Flow on PCA Scaffold

A novel vascular bioreactor system, developed to allow strain and shear stress to be applied to cells cultured on mock coronary arteries [129], was adapted for tubular lengths of the natural matrix scaffold of PCA. Four lengths of PCA, approximately 30 mm, were obtained. Two were decellularised for 15 hours and two were maintained in 0.9% saline as non-decellularised controls. Following sterilisation, the vessels were mounted and sealed in individual bioreactor chambers, see Figure 5.6.

The system consists of a CellMax® Bioreactor tubing system with serial pump which circulates culture medium from a reservoir to the bioreactor chamber. The reservoir maintains a mean pressure of 100 mmHg. The system was calibrated using a series of clamps to obtain a pulsatile flow of 120/80 mmHg. The luminal pressure was measured using a paediatric pressure catheter (Abbott, IL, USA) which was withdrawn after calibration to maintain system sterility. A filter on each bioreactor chamber allowed for gaseous exchange. Each reservoir contained 125 ml RPMI-1640 media supplemented with 10% FBS and 0.2% Primocin. Prior to connecting the reservoir, media was syringed through the tubing system to remove airlocks. The extraluminal space of the bioreactor chamber was filled with media and the entire system was placed in an incubator under normal conditions. The media was circulated from the reservoir by the bioreactor at a flow rate of 14 ml/min and at a pulse rate of 88 beats/min.

Images were recorded using a video extensometer every 5 days to obtain a measure of the external diameters. Additionally, diametrical compliance, C , was calculated using the inverse of Peterson's elastic modulus:

$$C = \frac{D_s - D_d}{D_d (P_s - P_d)} 10^4 \quad \% \text{mmHg}^{-1} \times 10^{-2} \quad (\text{Equation 5.1})$$

where, D_s and D_d are systolic and diastolic external diameters, and P_s and P_d are the systolic, 80 mmHg, and diastolic pressures, 120 mmHg.

The reservoir, tubing and extraluminal media was replaced every 5 days. The vessels were removed after 30 days and 2 mm sections were embedded in paraffin for

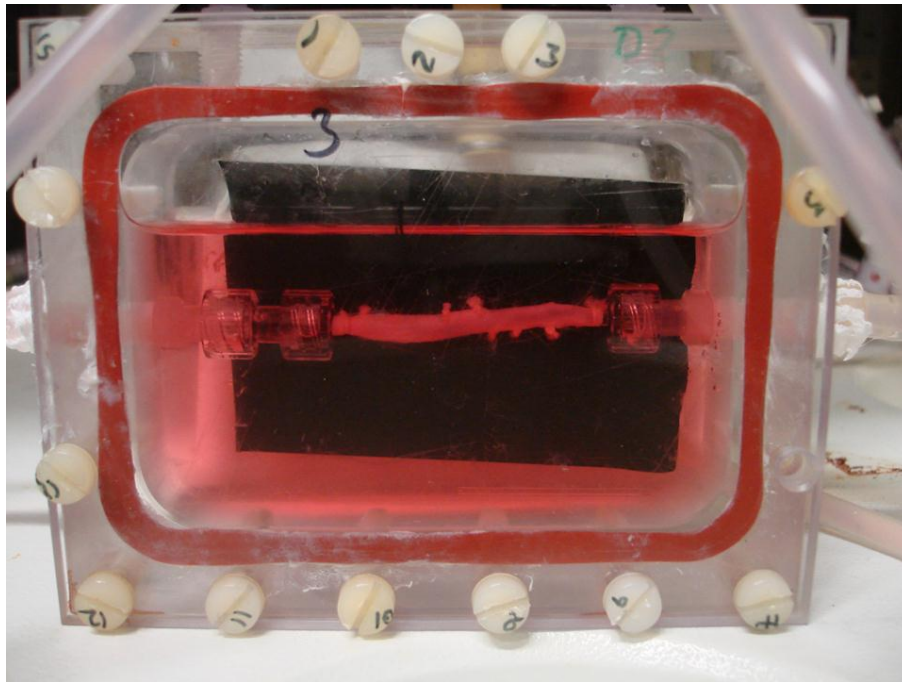


Figure 5.6: Bioreactor flow chamber. Tubular porcine coronary artery, with all branches ligated, mounted and sealed in bioreactor chamber and immersed in RPMI-1640 media.

histological analysis. Additionally, tensile tests were performed on 15 x 2.5 mm sections from each vessel according to the protocol in Section 4.1.2 (pg. 69).

5.1.9 Attachment of Other Cell Types

5.1.9.1 Mesenchymal Stem Cell Seeding

rMSCs were seeded to the abluminal surface of six 5 x 5 mm sections of 15 hour decellularised PCA in a 24-well plate. Sections were pretreated with 100% FBS by perfusion and soakage for 1 hour, respectively. rMSCs were seeded at a concentration of 5×10^4 cells/cm² to three sections and at a concentration of 2×10^5 cells/cm² to a further three sections. The sections were incubated in 50:50 Ham's and α -MEM media supplemented with 10% FBS and 0.2% Primocin. All sections were removed after 15 days and processed for histological and immunocytochemical analysis.

5.1.9.2 Human Coronary Artery Smooth Muscle Cell Seeding

HCASMC were seeded to the abluminal surface of six 5 x 5 mm sections of 15 hour decellularised PCA at a concentration of 2×10^4 cells/cm² in a 24-well plate. The cell-seeded sections were incubated in smooth muscle cell growth medium 2 and Supplement Mix with 10% FBS and 0.2% Primocin. Three sections were removed after 24 hours and 15 days of culture, respectively, and prepared for immunocytochemical analysis.

5.1.10 Histological and Immunohistochemical Analysis

Upon completion of each test, sections were repeatedly washed in sterile PBS to ensure any non-adhering cells were removed from the scaffold prior to fixing in 10% formalin for analysis.

4',6-diamidino-2-phenylindole (DAPI) analysis of the scaffold surface was used to determine the attachment of cells to the luminal and abluminal surfaces of decellularised PCA. Hematoxylin and Eosin (H&E) staining and DAPI staining of slide-mounted cross-sections of PCA established the degree of cell infiltration into the scaffold. Masson's Trichrome stain highlighted the presence of smooth muscle within the scaffold as a result of infiltrated cells. Modified Verhoeff Van Gieson Elastic stain determined the quality of elastin remaining in the sections. Sections were prepared and stained as described in Section 3.1.3 (pg. 47) and Section 3.1.4 (pg. 50).

5.1.11 Immunocytochemical Analysis

Cells were washed in PBS and fixed in 0.3% formaldehyde for 15 minutes at room temperature. Cells were washed twice in PBS and permeabilised in PBS containing 0.025% Triton X-100 (X100, Sigma-Aldrich) for 10 minutes. Cells were washed twice in PBS and were blocked in 5% bovine serum albumin (BSA)-PBS- 1% Tween (P5927 Sigma-Aldrich) for 2 hours at room temperature. Cells were incubated overnight at 4 °C in the appropriate primary antibody. Cells were washed in 1% Tween-PBS for 10 minutes. Cells were incubated for 1 hour at room temperature in the appropriate secondary antibody in the dark. Cells were rinsed three times in

PBS for 5 minutes and were stained with DAPI for 4 minutes. Cells were washed twice in PBS prior to mounting and imaging.

The primary antibody used for staining BAEC was endothelial Nitric Oxide Synthase 3 (eNOS, PA1-037, Thermo Scientific) and the secondary antibody was anti-rabbit IgG FITC conjugate (Invitrogen, Groningen, Netherlands). The primary antibody for staining BASMC was anti α -smooth muscle cell specific actin (α -SM actin, A5228, Sigma-Aldrich) and the secondary antibody was Alexa Fluor 546 conjugate (Invitrogen, Groningen, Netherlands).

5.1.11.1 FITC-Phalloidin Stain

Fluorescein Isothiocyanate Labeled Phalloidin (FITC-Phalloidin, P5282, Sigma-Aldrich) stains the actin filaments of smooth muscle cells. Briefly, PCA sections were washed three times in PBS for 5 minutes, fixed in 10% formalin for 5 minutes, washed extensively in PBS, permeabilised with 0.1% Triton X-100 in PBS, and washed three times in PBS for 5 minutes. Sections were then stained with 50 μ g/ml fluorescent phalloidin conjugate solution in PBS (containing 1% DMSO from original stock solution) for 40 minutes at room temperature in the dark. Sections were washed three times in PBS for 5 minutes, stained with DAPI for 15 minutes, and washed again in PBS prior to imaging.

5.1.12 Cell Count and Statistics

Cell counts were performed on sections of DAPI-stained cell-seeded decellularised PCA. This was performed using ImageJ 1.42 software (National Institutes of Health, MD, USA). The number of nuclei in a 300 x 300 μ m representative area of a section from a microscopic image was counted. The average cell count from three representative areas of each section was taken. A t-test was performed on the data sets to determine the p-value, where $p < 0.05$ was considered significant.

5.2 Results

Non-seeded decellularised tissue sections were used as controls for all experiments. There was no evidence of cells present in those sections by H&E analysis. The images presented are representative of all sections viewed.

5.2.1 BASMC Attachment and Infiltration of Scaffold

BASMC stain positively for the contractile protein α -SM actin, see Figure 5.7. BASMC were seeded to the luminal and abluminal surfaces of 9 hour decellularised PCAs to determine the suitability of the scaffold for cell attachment. BASMC were seeded at a concentration of 1×10^5 cells/cm². H&E and DAPI staining confirmed a confluent layer of cells attached to the luminal and abluminal surfaces of 9 hour decellularised PCA after 24 and 72 hours, respectively, see Figure 5.8. The internal elastic lamina, lining the luminal surface, acted as a barrier to cell infiltration. A minor degree of cell infiltration was observed on the abluminal surface after this short period of cell seeding.

BASMC were seeded to sections of PCA decellularised for 9, 12, and 15 hours to determine the decellularisation time which maximises cell infiltration. As the previous study indicated that cells would not infiltrate through the luminal surface, BASMC were seeded to the abluminal surface of decellularised PCA at a concentration of 5×10^4 cells/cm², see Figure 5.9. Cells are clearly visible throughout the medial and adventitial layers of H&E stained non-decellularised PCA after days 0, 5, and 10, see Figure 5.9 (A-C). However, with increasing culture time, cells near the lateral edges of the scaffold began to migrate from the scaffold, see Figure 5.9 (D-E). This was further evidenced by DAPI and Masson's Trichrome staining of cross-sections of non-decellularised PCA after 30 days, see Figure 5.10. The number of cell nuclei in media and adventitia of lateral edges of PCA areas was substantially reduced compared to central areas. Additionally, the quantity of smooth muscle in lateral edges of the sections was reduced as observed by Masson's Trichrome staining smooth muscle red. Pores appeared in the medial and adventitial layers of non-decellularised sections after 10 days and in some areas the media separated from the adventitia highlighting the degradation of the tissue with time, see Figure 5.9 (A-E).

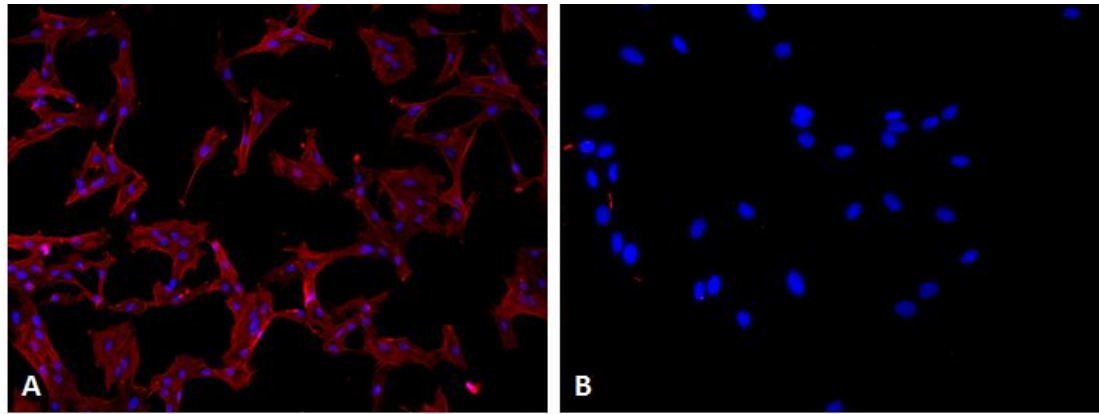


Figure 5.7: α -SM actin in bovine aortic smooth muscle cells. (A) Primary control and (B) no primary control. Magnification: 20x.

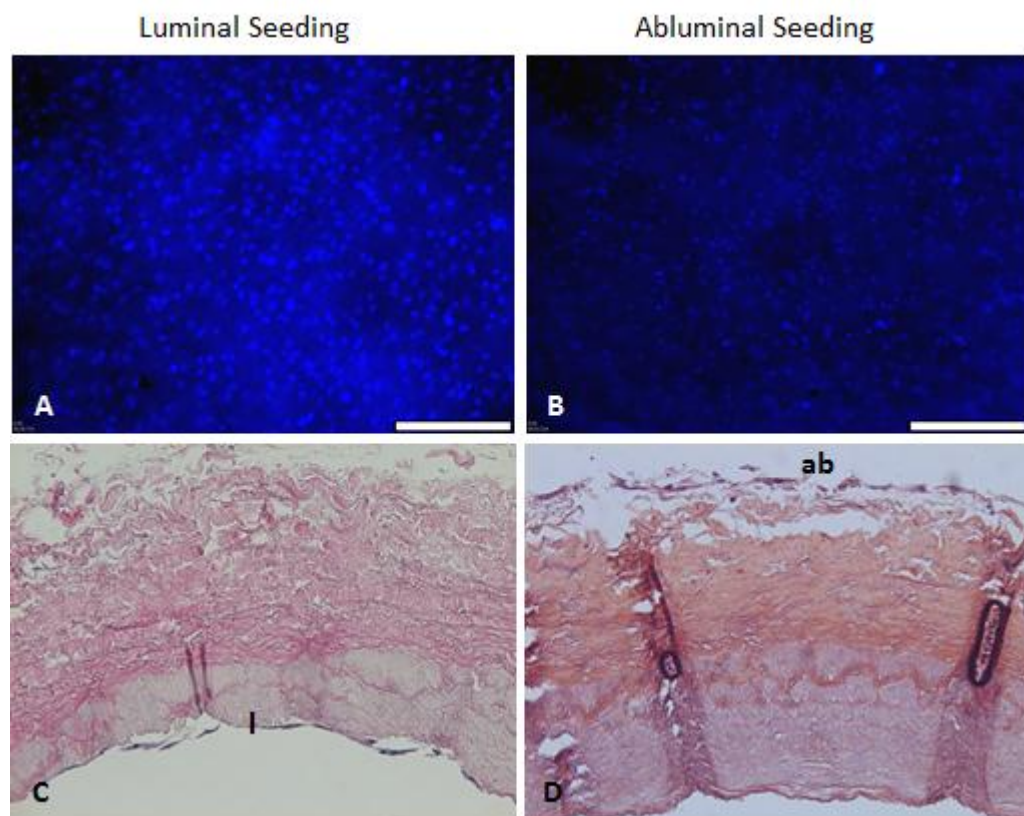


Figure 5.8: Luminal and abluminal BASMC-seeding of 9 hour decellularised porcine coronary arteries. DAPI staining of the (A) luminal and (B) abluminal surfaces after 24 hours and (C-D) H&E staining of the cross-sections after 72 hours. BASMC-seeding concentration of 1×10^5 cells/cm². l: luminal surface, ab: abluminal surface. Scale bar: 200 μ m, magnification: 10x.

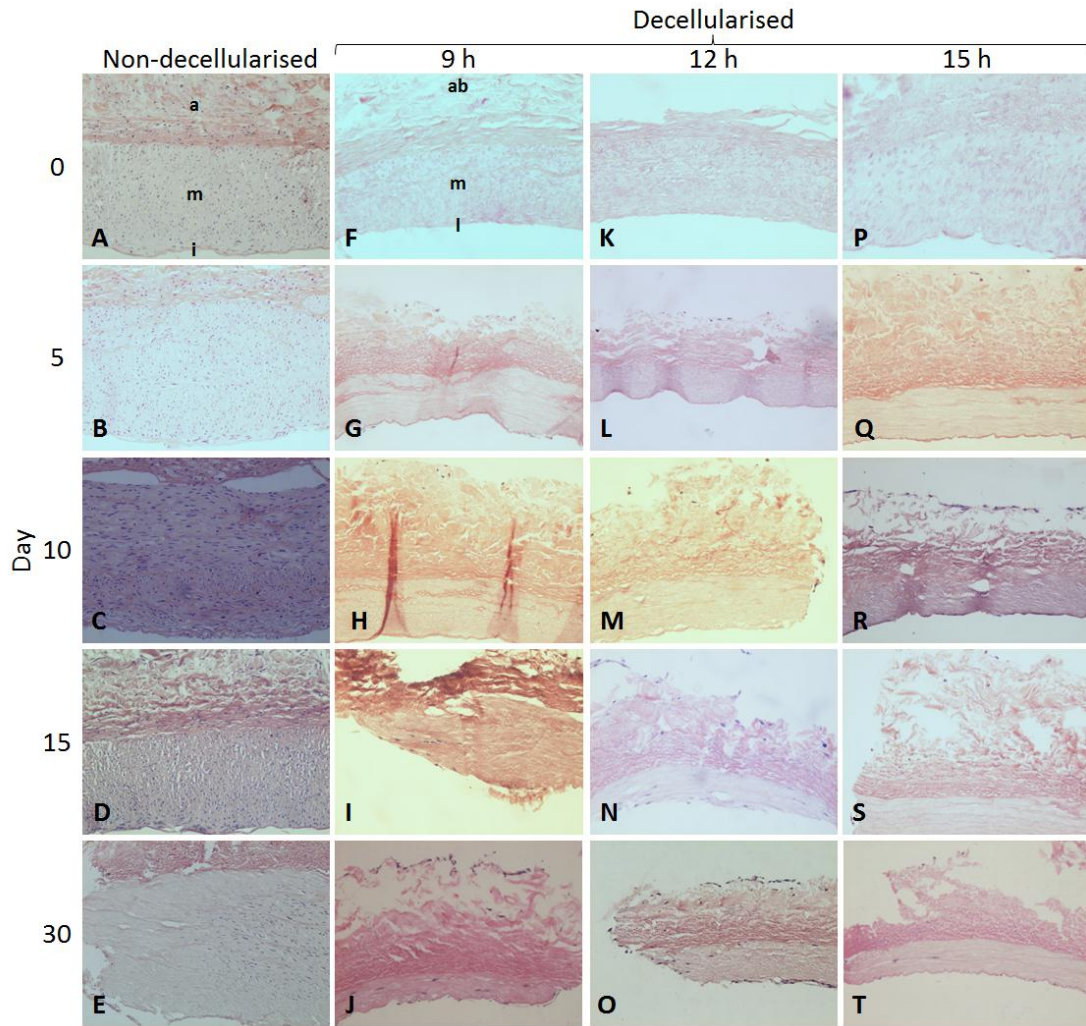


Figure 5.9: BASMC-seeding of the abluminal surfaces of decellularised porcine coronary arteries. H&E staining after 0, 5, 10, 15, and 30 days of the cross-sections of (A-E) non-decellularised porcine coronary arteries, and (F-J) 9 hour, (K-O) 12 hour, and (P-T) 15 hour decellularised porcine coronary arteries. BASMC-seeding concentration of 5×10^4 cells/cm². i: intima, m: media, a: adventitia, l: luminal surface, ab: abluminal surface. Magnification: 10x.

After 15 days, the non-seeded decellularised scaffolds, acting as controls, showed no significant degradation of the scaffold, see Figure 5.11.

There were no intact cell nuclei visible in 9, 12, and 15 hour decellularised sections at day 0, see Figure 5.9 (F, K, P). BASMC infiltration occurred on the abluminal and lateral edges of the scaffolds, increasing in number and distance infiltrated with increased culture time. After 5 days of culture a layer of BASMC was visible on the abluminal surface of all decellularised sections, see Figure 5.9 (G, L, Q). A degree of

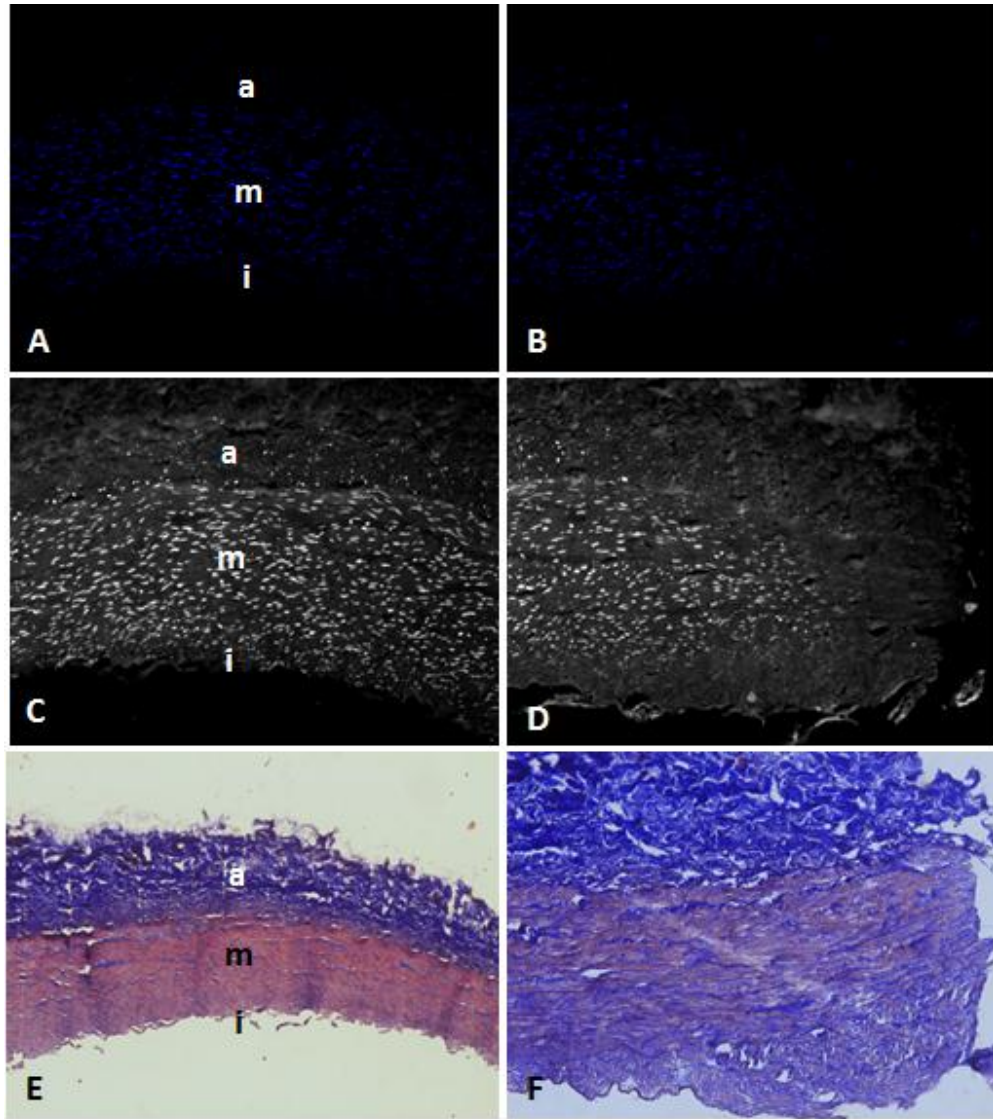


Figure 5.10: Migration of cells from non-decellularised sections after 30 days. (A-D) DAPI and (E-F) Masson's Trichrome staining of (A, C, E) central and (B, D, F) lateral cross-sections of non-decellularised porcine coronary arteries. i: intima, m: media, a: adventitia. Magnification: 10x.

infiltration into the abluminal surface was observed after 10 days, see Figure 5.9 (H, M, R). After 15 days of culture, BASMC infiltrated the lateral edges of the scaffolds and there was increased infiltration of the abluminal surfaces, see Figure 5.9 (I, N, S). After 45 days of culture, there was excellent infiltration along the length of the scaffolds in both the medial and the adventitial layers for all decellularisation times as evidenced by both H&E and DAPI staining, see Figure 5.12 (A-O). The greatest infiltration occurred in 15 hour decellularised sections. Additionally, Masson's

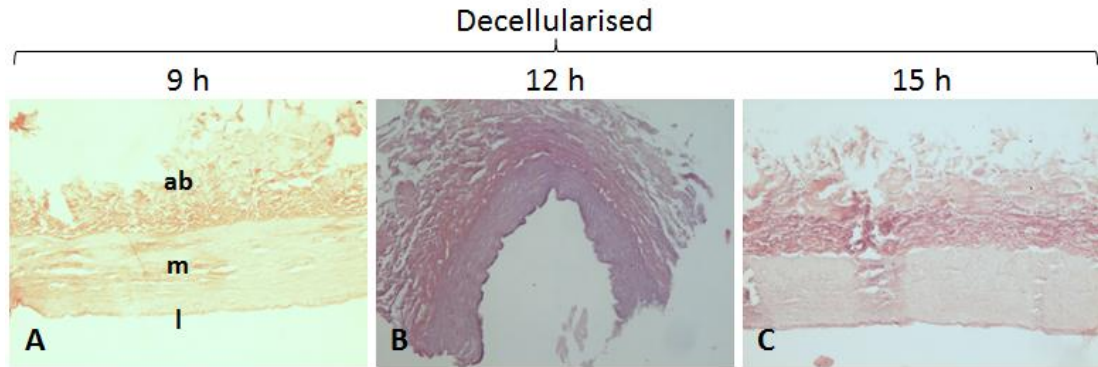


Figure 5.11: Decellularised controls after 15 days culture. H&E staining of the cross-sections of non-seeded (A) 9 hour, (B) 12 hour and (C) 15 hour decellularised PCA. l: luminal surface, m: media, ab: abluminal surface. Magnification: 10x.

Trichrome staining highlighted the presence of smooth muscle within the medial layer as a result of BASMC infiltration, see Figure 5.12 (P-R).

15 hour decellularised sections showed the greatest degree of BASMC infiltration, therefore, further cell seeding and biomechanical experiments were performed using this scaffold. The effect of increasing the cell concentration was examined. DAPI staining showed an increase from day 5 to day 15 in the number of BASMC on the abluminal surface for both high (2×10^5 cells/cm²) and low (5×10^4 cells/cm²) cell-seeding concentrations, see Figure 5.13. The 5 x 5 mm sections were not fixed to the base of the 24-well plates. Therefore, in some instances they did not remain flat for the duration of the test. As a result, for these sections a non-uniform dispersion of cells on the abluminal surface, where the cells were initially seeded, was observed. Additionally, cells attached to the luminal surface due to the curling of these sections. However, for sections that remained flat for the duration of the test, after day 5 there were no cells in central sections of the luminal surface, yet there were clusters of BASMC at the lateral edges, see Figure 5.14 (A - D). By day 15 BASMC had migrated onto and across the luminal surface and were observed in central areas although no infiltration occurred through the luminal surface, see Figure 5.14 (E, G).

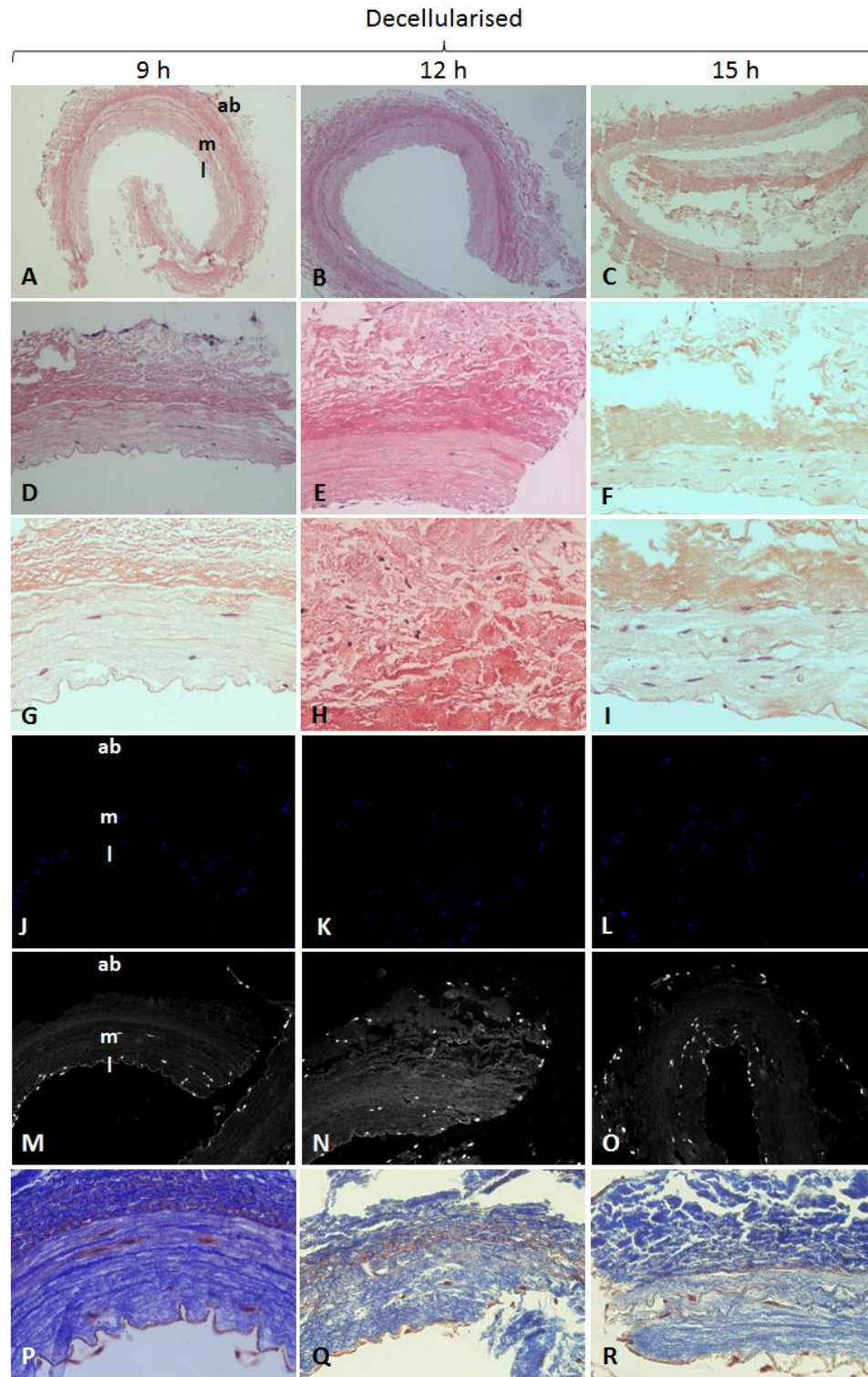


Figure 5.12: Infiltration of BASMC into decellularised porcine coronary arteries after 45 days. (A-I) H&E, (J-O) DAPI, and (P-R) Masson's Trichrome staining of the cross-sections of 9 hour, 12 hour, and 15 hour decellularised porcine coronary arteries. l: luminal surface, m: media, ab: abluminal surface. Magnification: (A-C) 4x, (D-F, J-R) 10x, (G-I) 20x.

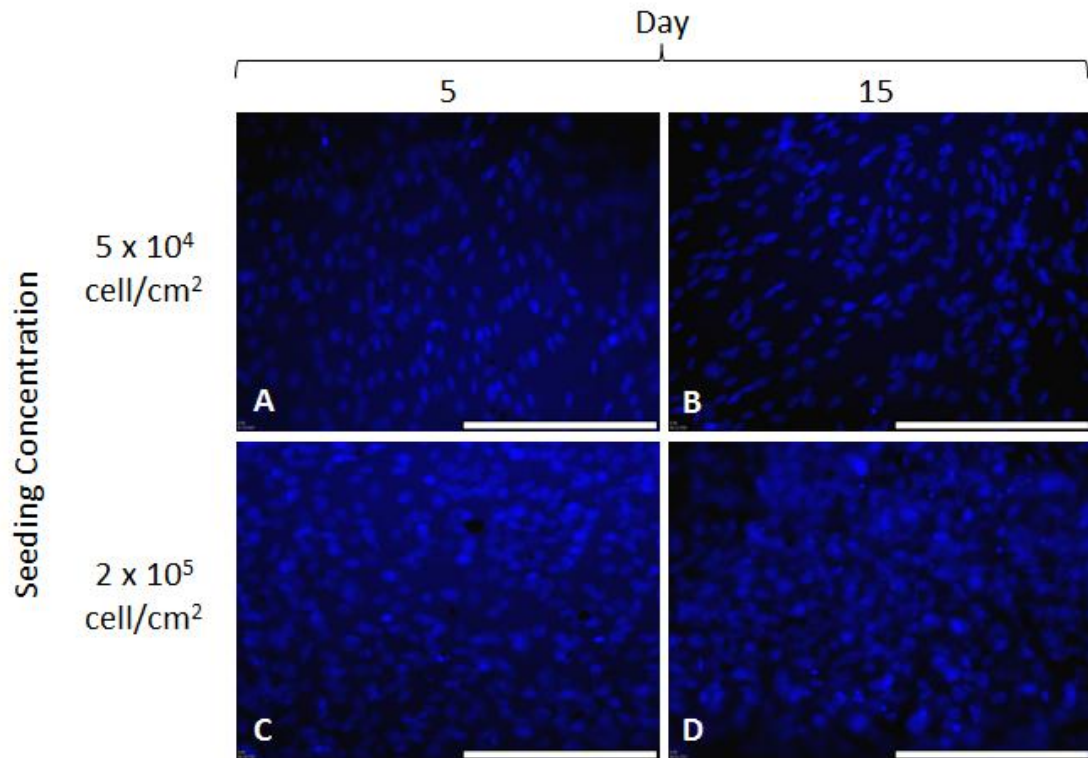


Figure 5.13: High (2×10^5 cells/cm²) and low (5×10^4 cells/cm²) cell-seeding concentrations. DAPI staining of the abluminal surface of BASMC-seeded PCA after (A, C) 5 days and (B, D) 15 days culture. Scale bar: 200 μ m.

H&E staining after 5 days of culture verified a greater number of cells attached to the higher concentration-seeded scaffolds in comparison to the lower concentration-seeded sections, see Figure 5.15. BASMC attached to the abluminal and lateral edges of the scaffolds although there was no infiltration visible at this stage. Clusters of cells occurred on the higher seeding concentration sections, in particular near the edges of the sections, whilst a confluent layer of cells formed on the abluminal surface, see Figure 5.15 (D-F). In some areas the cells were layered as also noted in the DAPI analysis.

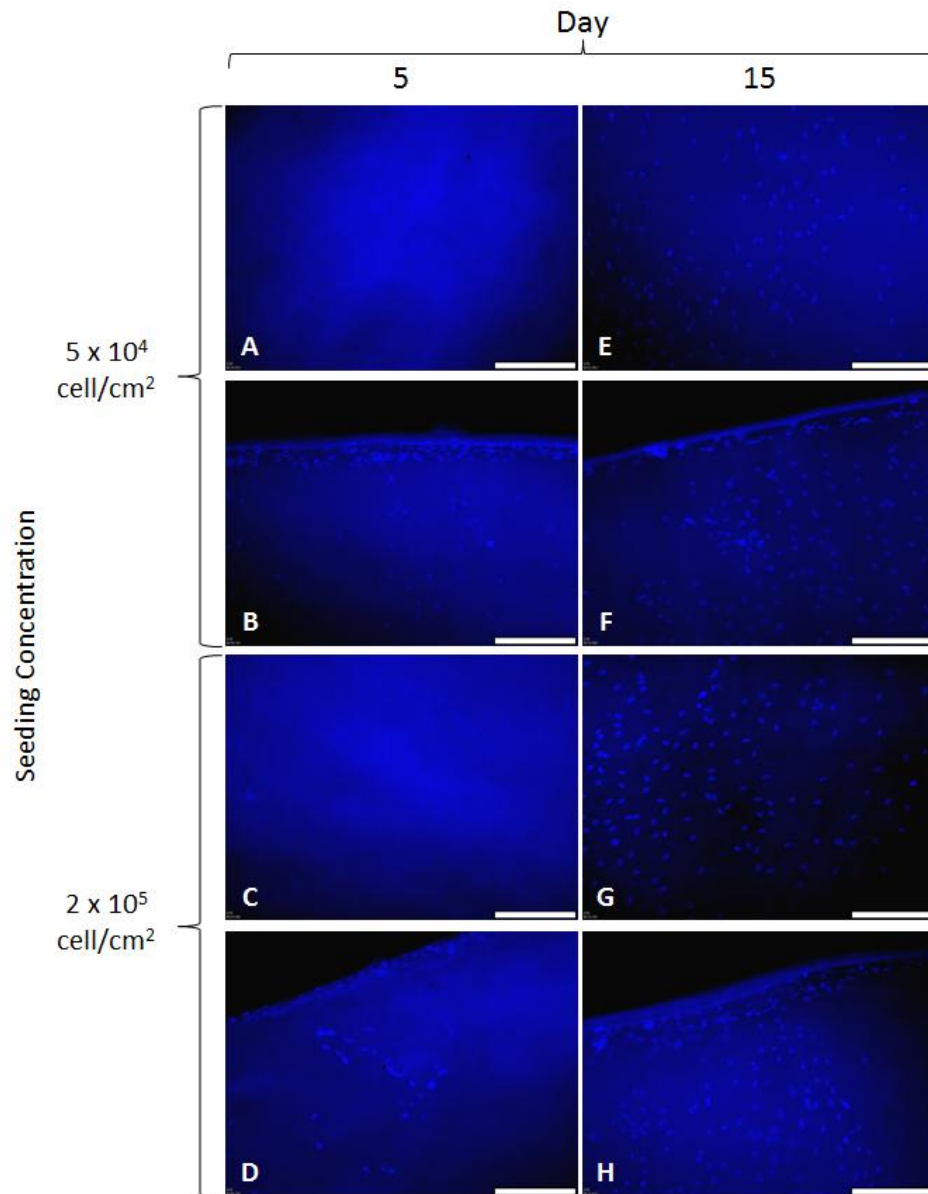


Figure 5.14: Migration of BASMC across the luminal surface of abluminally-seeded 15 hour decellularised porcine coronary arteries. DAPI staining of (A, C, E, G) central and (B, D, F, H) lateral areas of the luminal surface of porcine coronary arteries seeded at (A-B, E-F) a low concentration of 5×10^4 cells/cm² and (C-D, G-H) a high concentration of 2×10^5 cells/cm² after 5 days and 15 days culture. Scale bar: 200 μ m.

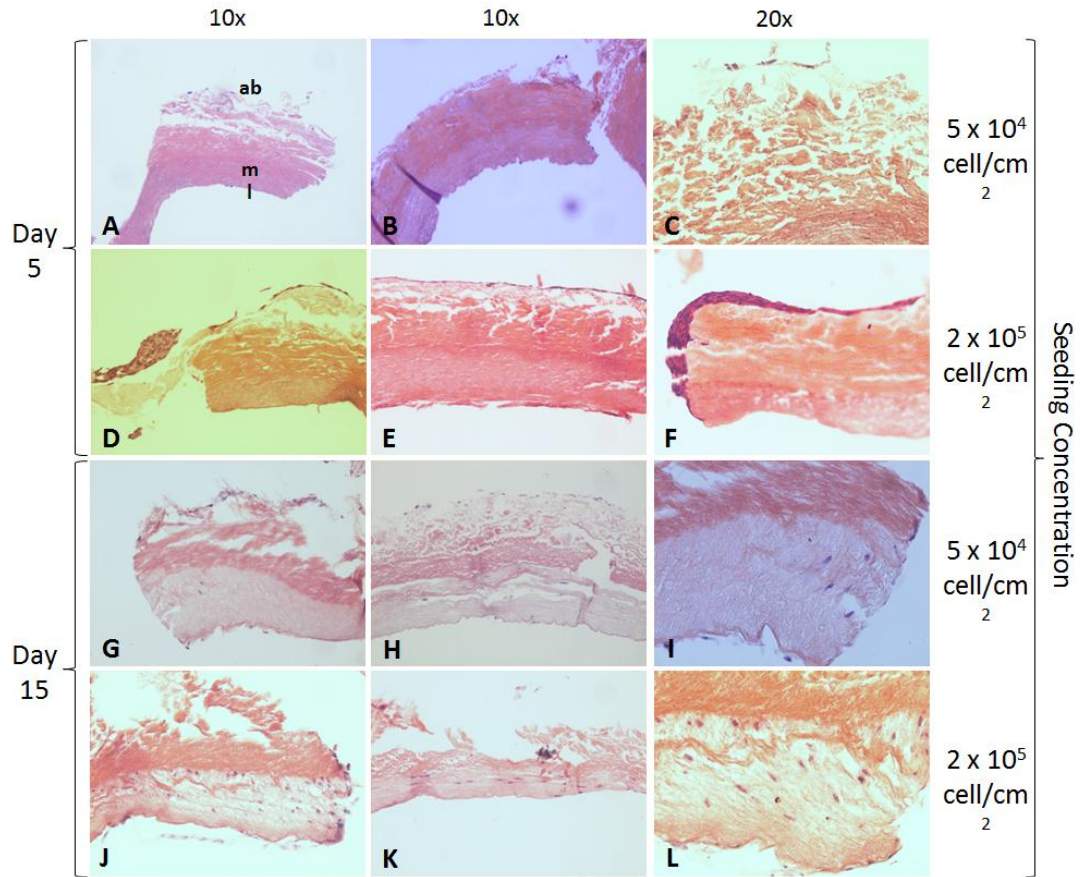


Figure 5.15: BASMC attachment and infiltration of 15 hour decellularised porcine coronary arteries seeded with low and high cell concentrations after 5 and 15 days. Representative images of H&E staining of the cross-sections of different segments seeded at concentrations of (A-C, G-I) 5×10^4 cells/cm² and (D-F, J-L) 2×10^5 cells/cm² after 5 days and 15 days culture. l: luminal surface, m: media, ab: abluminal surface. Magnification: (A-B, D-E, G-H, J-K) 10x, (C, F, I, L) 20x.

5.2.2 Scaffold Endothelialisation

BAEC stain positively for the enzyme eNOS, see Figure 5.16. BAEC were seeded to the luminal surface of 15 hour decellularised scaffolds at a concentration of 2×10^4 cells/cm² to determine their ability to attach and form a confluent monolayer. H&E and DAPI staining confirmed the presence of BAEC on the luminal surface after 1 day and the formation of an almost confluent layer after 10 days, see Figure 5.17 and Figure 5.18. No cell infiltration was observed through the internal elastic lamina. A low number of cells attached to the luminal surface after 1 day indicating that more time was required for BAEC to attach to the surface. However, after 10 days of culture there was a statistically significant higher concentration of BAEC on the luminal surface, see Figure 5.19. H&E staining showed that the contact-inhibited BAEC attached to the luminal surface to form a monolayer, see Figure 5.18 (A-B). In some areas the endothelial layer appeared to have detached from the luminal surface. This was most likely due to the harsh process involved in the embedding procedure as the DAPI staining indicated that BAEC adhered to the surface.

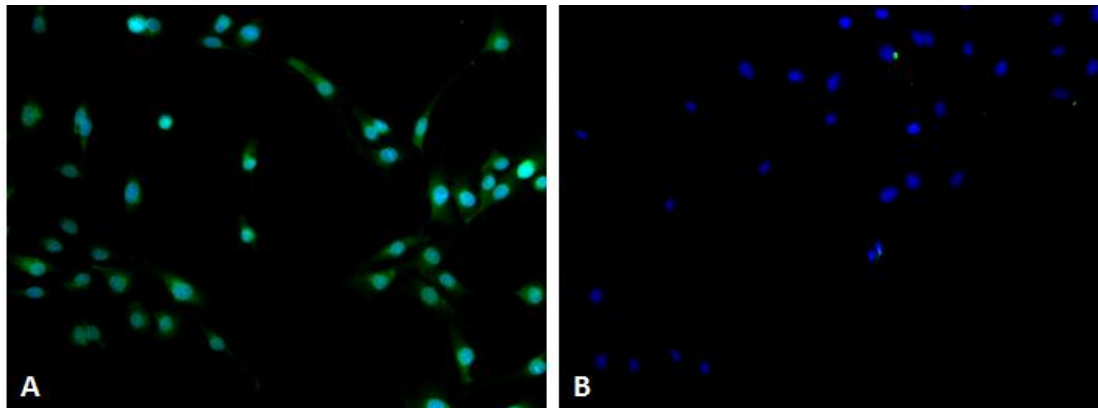


Figure 5.16: eNOS in bovine aortic endothelial cells. (A) Primary control and (B) no primary control. Magnification: 20x.

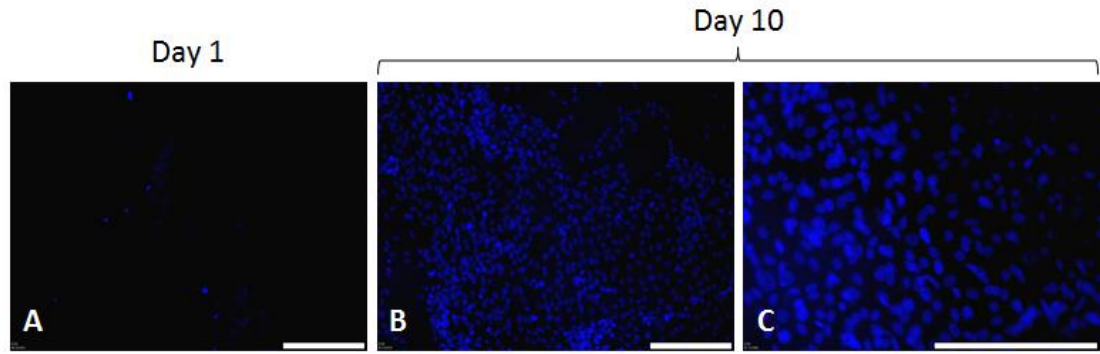


Figure 5.17: Endothelial cell attachment to the luminal surface of PCA scaffold. DAPI staining of 15 hour decellularised PCA seeded with BAEC at a concentration of 2×10^4 cells/cm² after (A) 24 hours and (B-C) 10 days. Scale bar: 200 μ m.

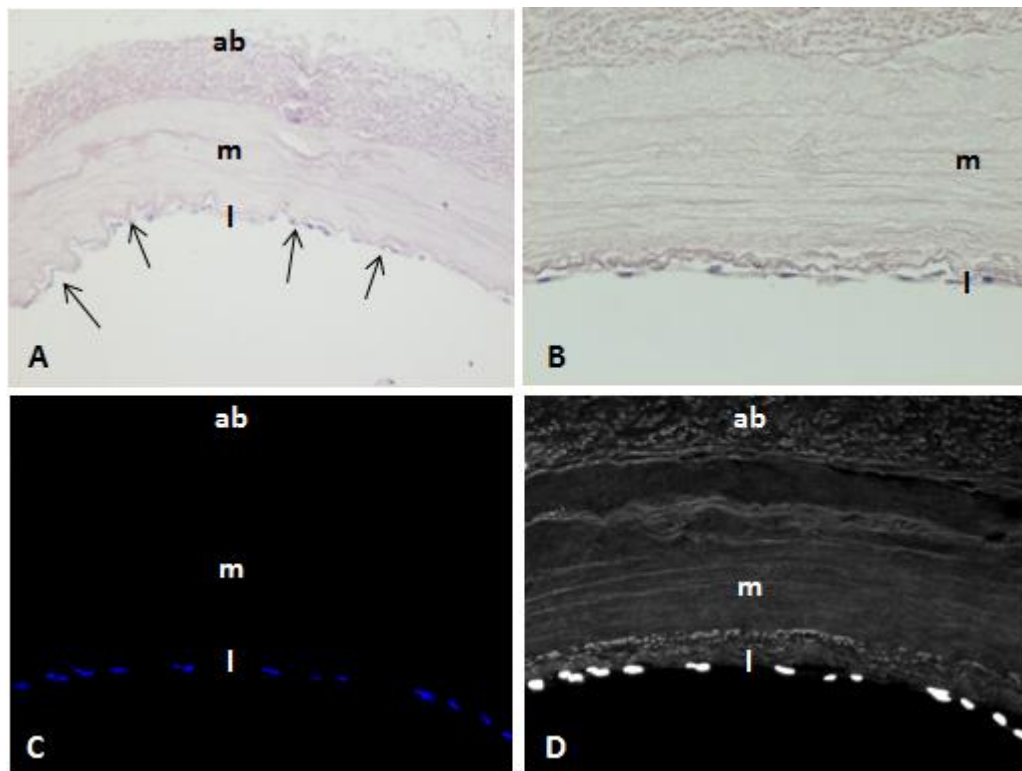


Figure 5.18: Endothelial cell attachment to the luminal surface of 15 hour decellularised scaffold after 10 days. (A-B) H&E and (C-D) DAPI staining of BAEC-seeded 15 hour decellularised PCA. l: luminal surface, m: media, ab: abluminal surface. Magnification: (A) 10x, (B-D) 20x.

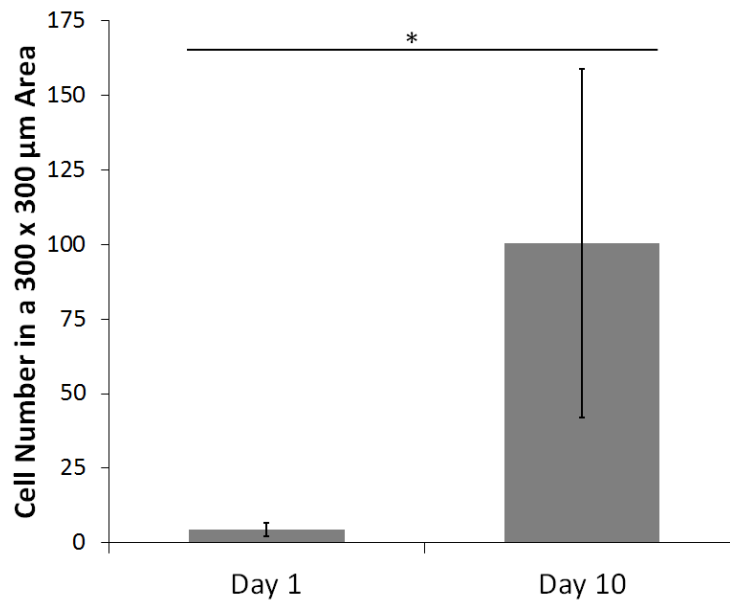


Figure 5.19: Endothelial cell count after 1 and 10 days culture on the luminal surface of 15 hour decellularised porcine coronary arteries.

5.2.3 Seeding of 3D Tubular Scaffold

Representative images from distal, central, and proximal sections of the 40 mm length of PCA scaffold are shown in Figure 5.20. DAPI staining demonstrated an almost confluent layer of BASMC adhered to the luminal and abluminal surfaces of the scaffold after 8 days, see Figure 5.20 (A-F). H&E staining of cross-sections of the scaffold also indicated the attachment of BASMC on both luminal and abluminal surfaces along the 40 mm length, see Figure 5.20 (G-O). By 8 days, although infiltration had occurred via the lateral edges of sections 1 and 7, the most distal and proximal sections of the arterial segment, see Figure 5.20 (G, I, M, O), it did not extend beyond the distal and proximal ends of the scaffold, see Figure 5.20 (H, K, N). There was no infiltration through the luminal surface as expected, whilst a degree of infiltration from the abluminal surface was observed.

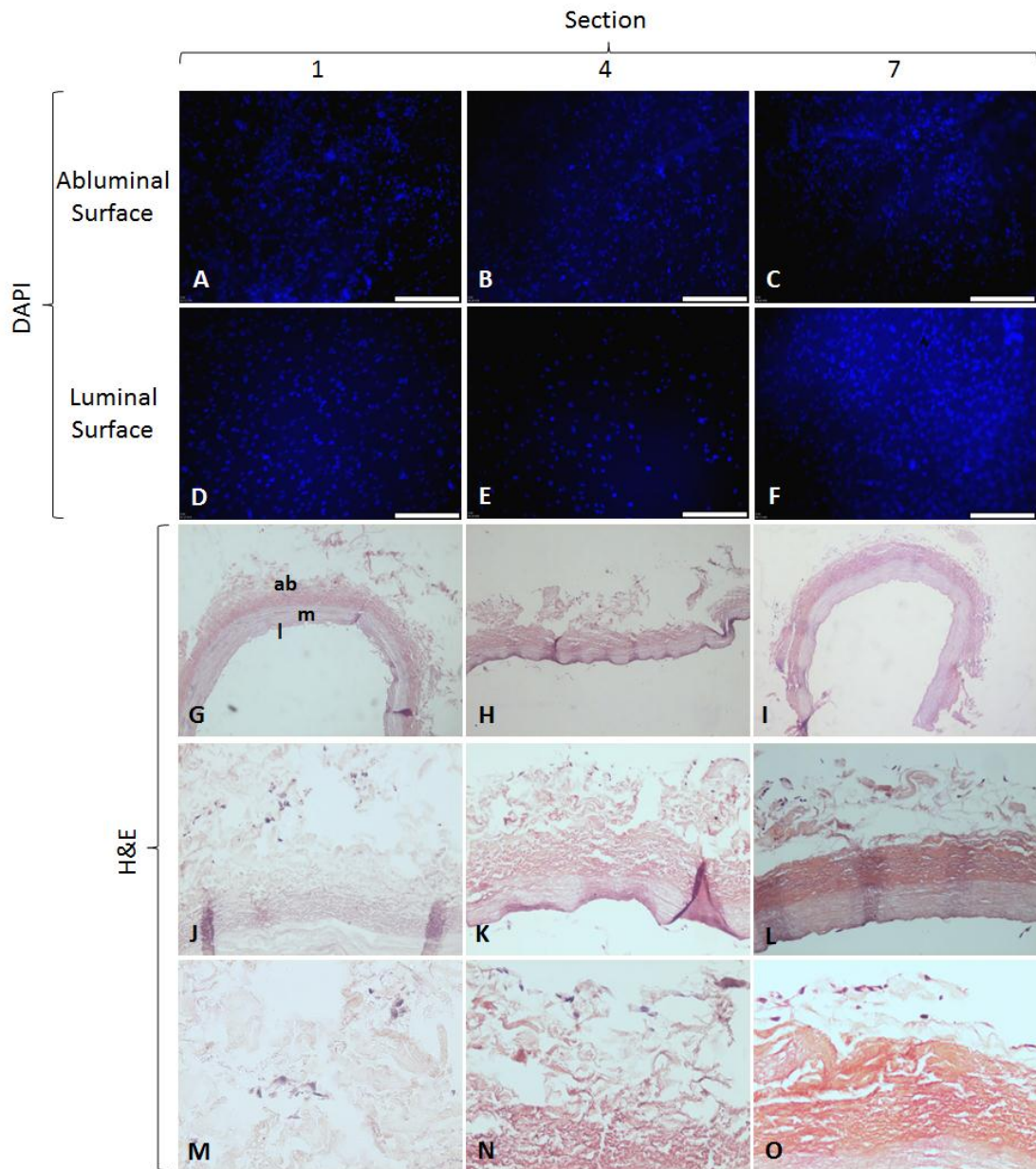


Figure 5.20: 40 mm length of BASMC-seeded 15 hour decellularised 3D tubular porcine coronary artery. (A-F) DAPI and (G-O) H&E staining of (A, D, G, J, M) proximal, (B, E, H, K, N) central and (C, F, I, L, O) distal sections. l: luminal surface, m: media, ab: abluminal surface. Scale bar: 200 μ m. Magnification: (G-I) 4x, (A-F, J-L) 10x, (M-O) 20x.

5.2.4 Effect of Uniaxial Cyclic Strain on BASMC-seeded Scaffold

BASMC were seeded to the luminal surface of 9 hour decellularised PCA at a concentration of $1.25 \times 10^5 \text{ cm}^2$ for a total of 96 hours. To determine if there was cell alignment as a result of cyclic uniaxial strain, cells were stained with FITC-Phalloidin and DAPI. Static sections displayed a dispersed distribution of cells on the luminal surface, see Figure 5.21 (A, C). An even distribution of BASMC was observed on the luminal surface of sections which underwent 72 hours of cyclic uniaxial strain, see Figure 5.21 (B, D). Furthermore, the alignment of BASMC actin filaments at approximately 45° to the direction of stimulation was evident, see Figure 5.21 (B, D). There was no alignment of cells seeded to static sections. There were no cells visible on the surface of non-seeded sections, see Figure 5.21 (E-F).

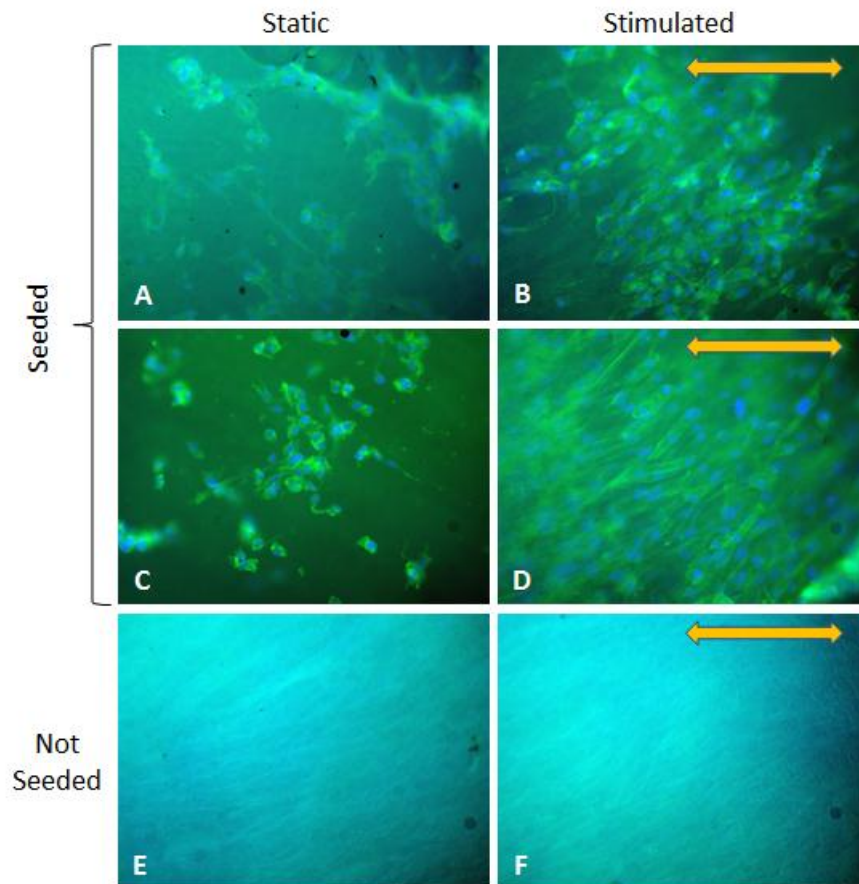


Figure 5.21: DAPI and FITC-Phalloidin staining of the luminal surface of 9 hour decellularised porcine coronary arteries following 72 hours in (A, C, E) static and (B, D, F) dynamic conditions. Arrows indicate the direction of the applied cyclic uniaxial strain. Magnification: (A-B, E- F) 10x, (C-D) 20x.

5.2.5 Biomechanical Analysis of BASMC-seeded Scaffold

Non-decellularised, decellularised, and BASMC-seeded decellularised sections all exhibited the typical J-shaped curve associated with soft tissue, see Figure 5.22. The variability of soft tissue was evident in the stress-strain response, in particular for seeded and non-seeded decellularised sections.

The final slopes and final x-intercepts of the stress-strain response was analysed for all sections using the method described in Section 4.1.2 (pg. 69), see Table 5.1 and Figure 5.23. Seeded decellularised sections were less distensible than non-seeded decellularised sections. However, the high variability in the stress-strain response of both decellularised and seeded decellularised sections resulted in no statistical significance. The final x-intercept of non-decellularised sections occurred at a significantly higher strain than seeded decellularised sections. The slopes of the final curve, associated with collagen recruitment, were equivalent for non-seeded decellularised and seeded decellularised sections. Although, these final slopes exhibited statistically significant stiffer behaviour compared to non-decellularised sections. Additionally, there was no statistical significance between the ultimate tensile strength of all three categories. A comparison of seeded and non-seeded decellularised sections obtained from the same arteries is shown in Figure 5.24, demonstrating, in the majority of cases, the less distensible seeded sections in comparison to non-seeded sections.

When viewed under a microscope during the course of the experiment, cells of the 5 x 15 mm non-decellularised sections were observed migrating from the tissue after 9 days. H&E staining of these sections fixed after 30 days confirmed the migration of cells from the lateral edges of the tissue while the interior of the tissue remained populated, see Figure 5.25 (A, D). While the number of cells observed is low compared to non-decellularised tissue, BASMC infiltrated the scaffold of the seeded decellularised PCA in both the medial and the adventitial layers, see Figure 5.25 (C, F). Sections were embedded and stained following the tensile tests, therefore, pores were visible throughout the thickness of all sections due to the sections being tested to failure prior to fixing. Modified Verhoeff Van Gieson staining of the sections highlight the presence of elastin in the tissue, see Figure 5.25 (G-I). The quality of

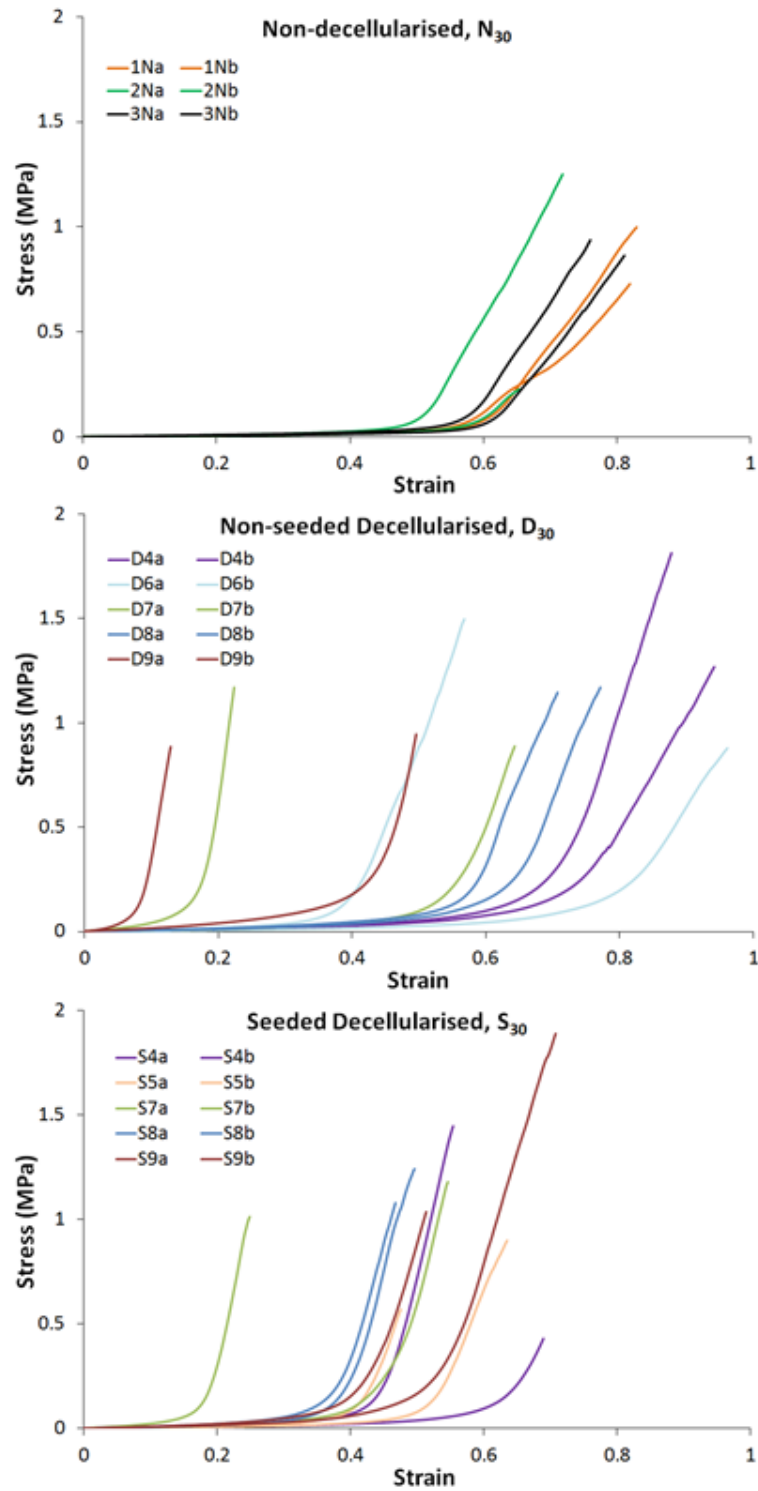


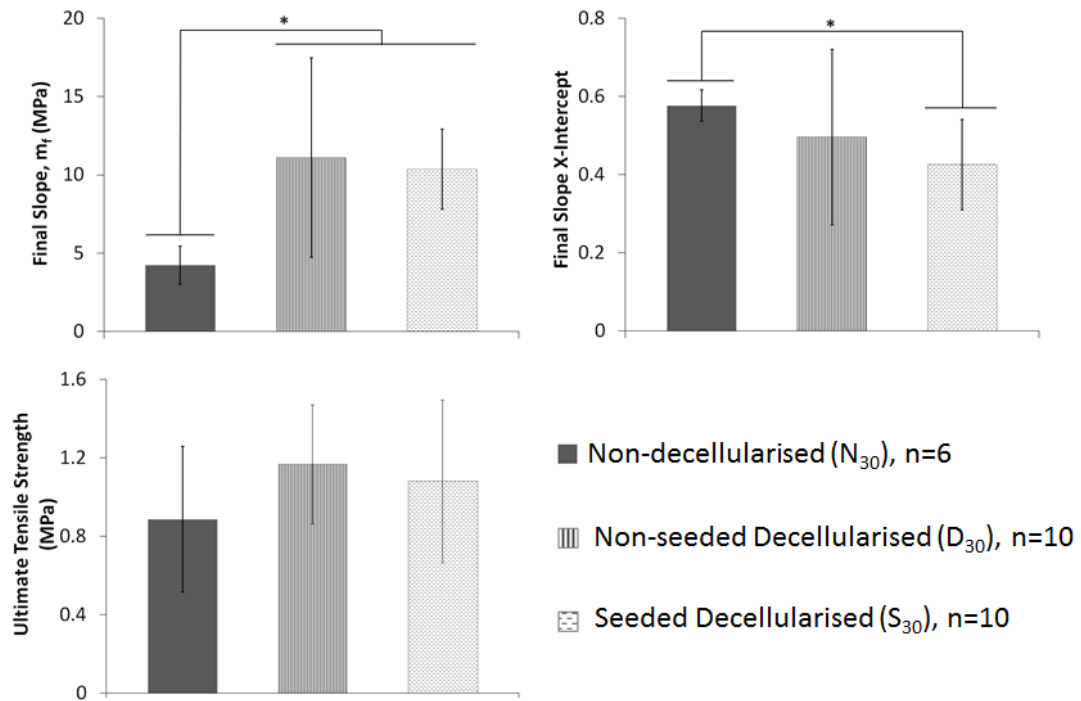
Figure 5.22: Uniaxial tensile tests of non-decellularised (N_{30}), 15 hour decellularised (D_{30}), and BASMC-seeded 15 hour decellularised (S_{30}) sections of porcine coronary arteries after 30 days of static culture.

elastin in the adventitia of non-decellularised sections appeared diminished compared to seeded and non-seeded decellularised sections.

Table 5.1: Uniaxial tensile test data of 30 day cultured non-decellularised (N_{30}), decellularised (D_{30}), and BASMC-seeded (S_{30}) porcine coronary arteries after 30 days of static culture.

	Final Slope, m_f (MPa)	Final X-Intercept	Ultimate Tensile Strength (MPa)	Gauge Length Before Pre-conditioning (mm)	Gauge Length After Pre-conditioning (mm)
N_{30} , n=6	4.23 ± 1.23	0.58 ± 0.04	0.88 ± 0.37	10.03 ± 0.25	11.44 ± 0.28
D_{30} , n=10	$11.10 \pm 6.38^*$	0.50 ± 0.23	1.17 ± 0.30	9.96 ± 0.13	11.94 ± 2.01
S_{30} , n=10	$10.34 \pm 2.56^*$	$0.42 \pm 0.12^*$	1.08 ± 0.41	10.08 ± 0.22	$12.47 \pm 1.34^*$
Significance, * $p < 0.05$ from non-decellularised (N_{30}) sections					

Stress-Strain Analysis



Dimension Analysis

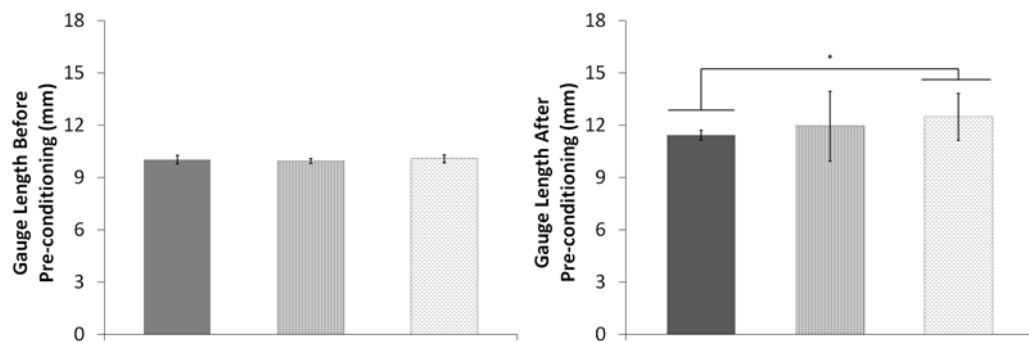


Figure 5.23: Uniaxial tensile data for non-decellularised (N_{30}), 15 hour decellularised (D_{30}), and 15 hour BASMC-seeded decellularised (S_{30}) sections of porcine coronary arteries after 30 days of static culture. Significance, * $p < 0.05$.

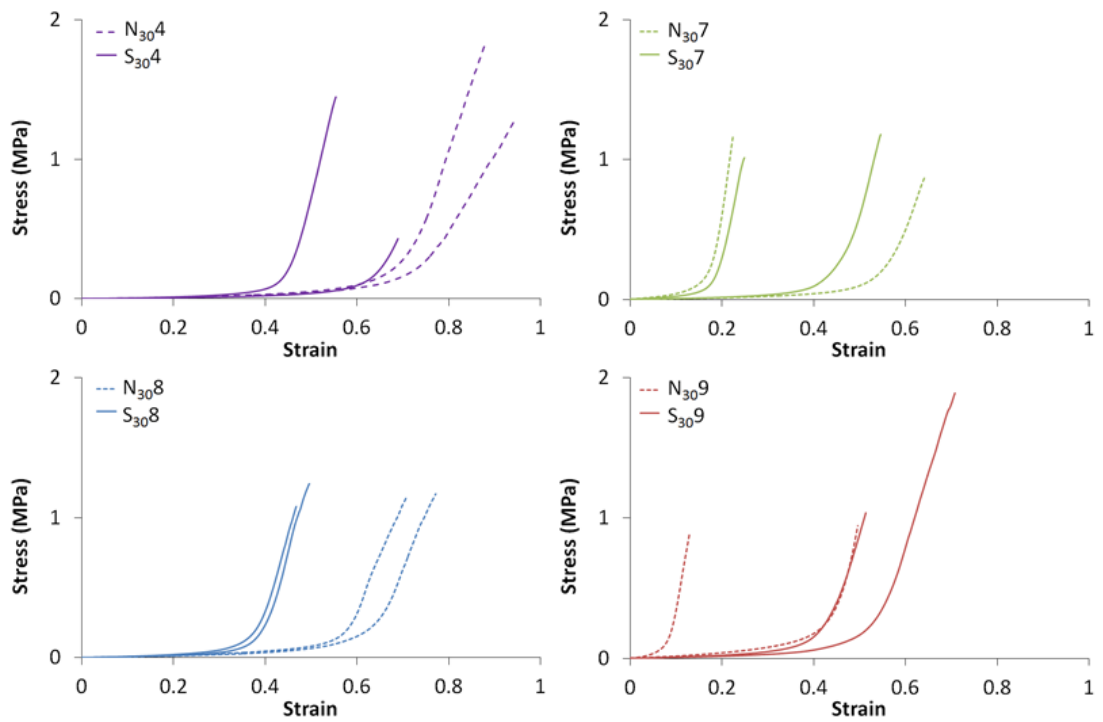


Figure 5.24: Comparison of uniaxial tensile tests performed on consecutive sections of non-seeded (N) and seeded (S) decellularised after 30 days of static culture.

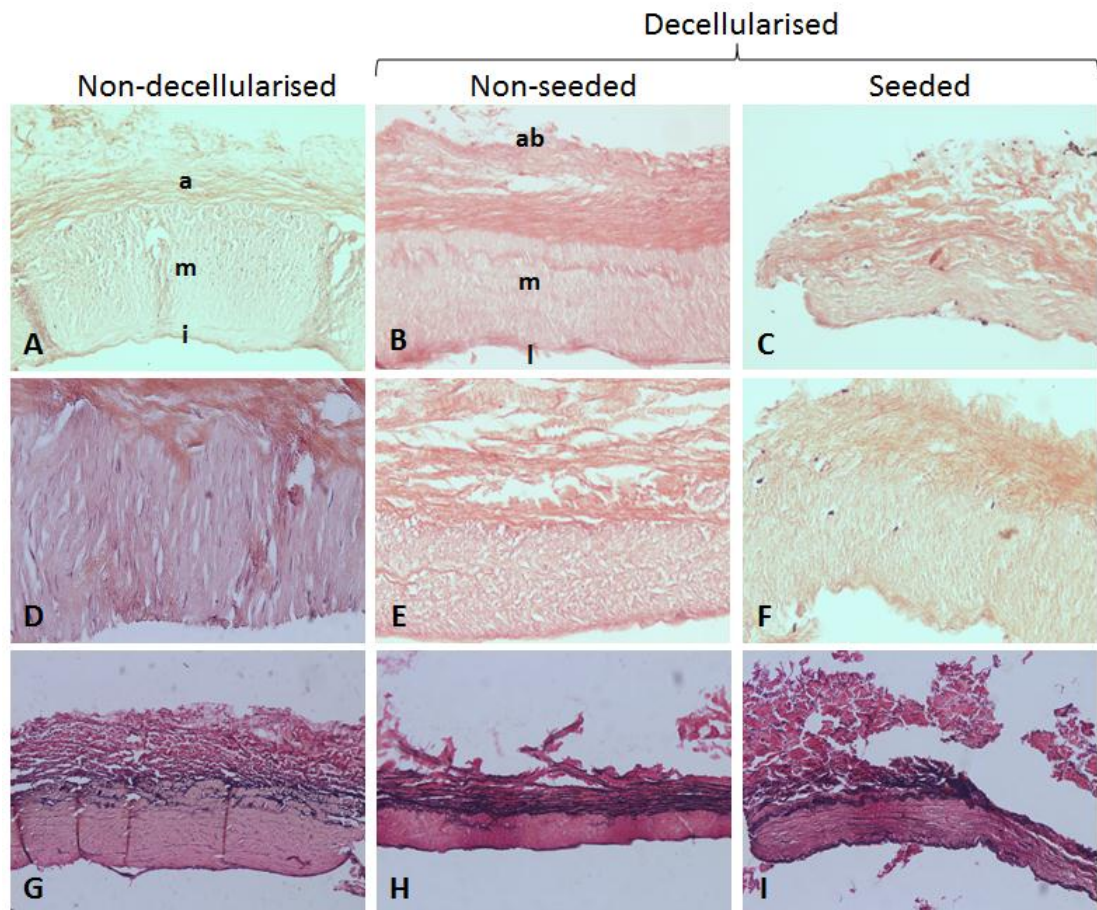


Figure 5.25: Sections of (A, D, G) non-decellularised, (B, E, H) non-seeded decellularised, and (C, F, I) seeded decellularised porcine coronary arteries after 30 days static culture. (A-F) H&E and (G-I) Modified Verhoeff Van Gieson stained sections after uniaxial tensile testing. i: intima, m: media, a: adventitia, l: luminal surface, ab: abluminal surface. Magnification: (A-C, G-I) 10x, (D-E) 20x.

5.2.6 Effect of Pulsatile Flow on PCA Scaffold

Both non-decellularised vessels became contaminated after 5 days and 20 days, respectively, and were removed from the experiment. Both decellularised sections were removed after 30 days of pulsatile flow at physiological pressure. There were minor leakages throughout the course of the experiment for all segments, although all branches remained ligated. There was no indication of aneurysm formation in any vessel, see Figure 5.26 and Figure 5.27. Due to the design of the bioreactor chambers and the luer connectors on which the arteries were mounted, it was not possible to apply a known pre-stretch to the vessels. As a result, the pre-stretch applied to decellularised artery B was insufficient and caused bending of the vessel upon pressurisation. However, the other three vessels remained straight when pressurised. The external diameter at 100 mmHg was measured for each vessel. There was a significant decrease in the external diameter of non-decellularised artery B at day 20 which corresponded to contamination of the vessel, see Figure 5.26. Additionally, both decellularised vessels also exhibited a decrease in external diameter after day 15, see Figure 5.27.

With the exception of the compliance of decellularised vessel A at day 5 and decellularised vessel B at day 10, there is a steady increase in the compliance of all vessels with time, see Figure 5.28 (A). Large increases occurred after day 15 corresponding to the decrease in external diameter exhibited in all vessels.

Uniaxial tensile tests of the decellularised sections were performed following 30 days in the bioreactor. The typical J-shaped stress-strain response associated with soft tissue was observed for both vessels, see Figure 5.28 (B). Final slopes of 8.53 MPa and 6.86 MPa were calculated for decellularised vessel A and decellularised vessel B, respectively.

The decellularised PCA remained intact for the duration of the experiment, see Figure 5.29. However, H&E staining indicated loose, porous layers, particularly in the adventitia. Additionally, Modified Verhoeff Van Gieson staining indicated a breakdown of the internal elastic lamina in regions following 30 days of pulsatile flow, see Figure 5.29 (E-F).

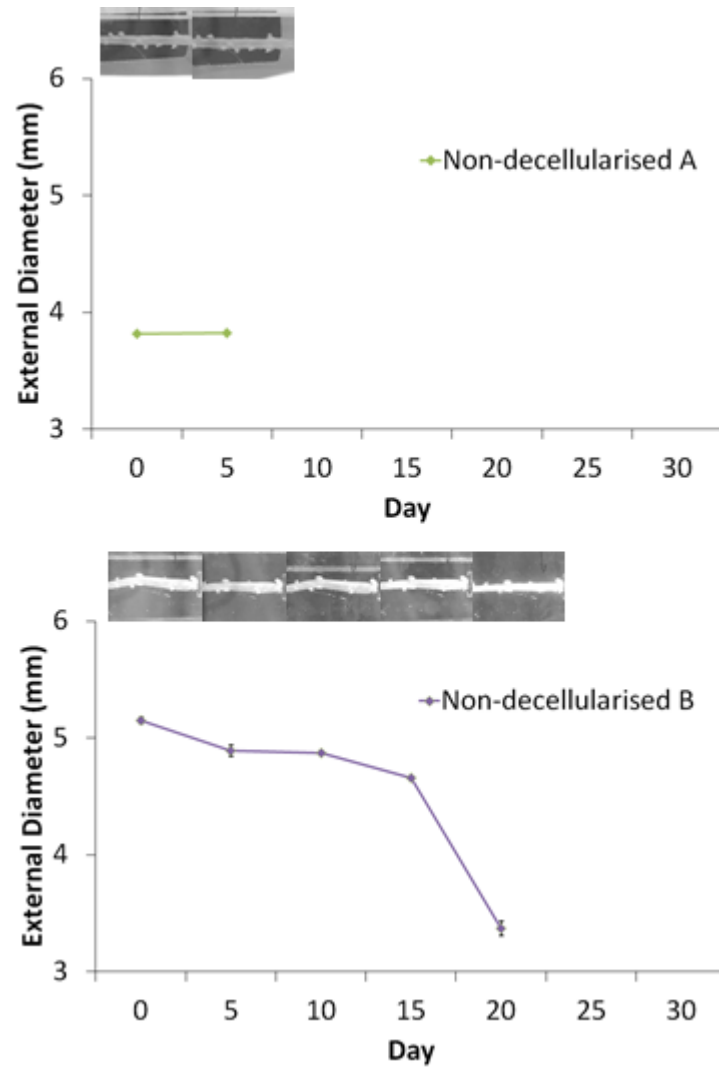


Figure 5.26: Biomechanical analysis of the effect of pulsatile flow at physiological pressures on non-decellularised porcine coronary arteries. External diameter at 100 mmHg and corresponding images for each time point.

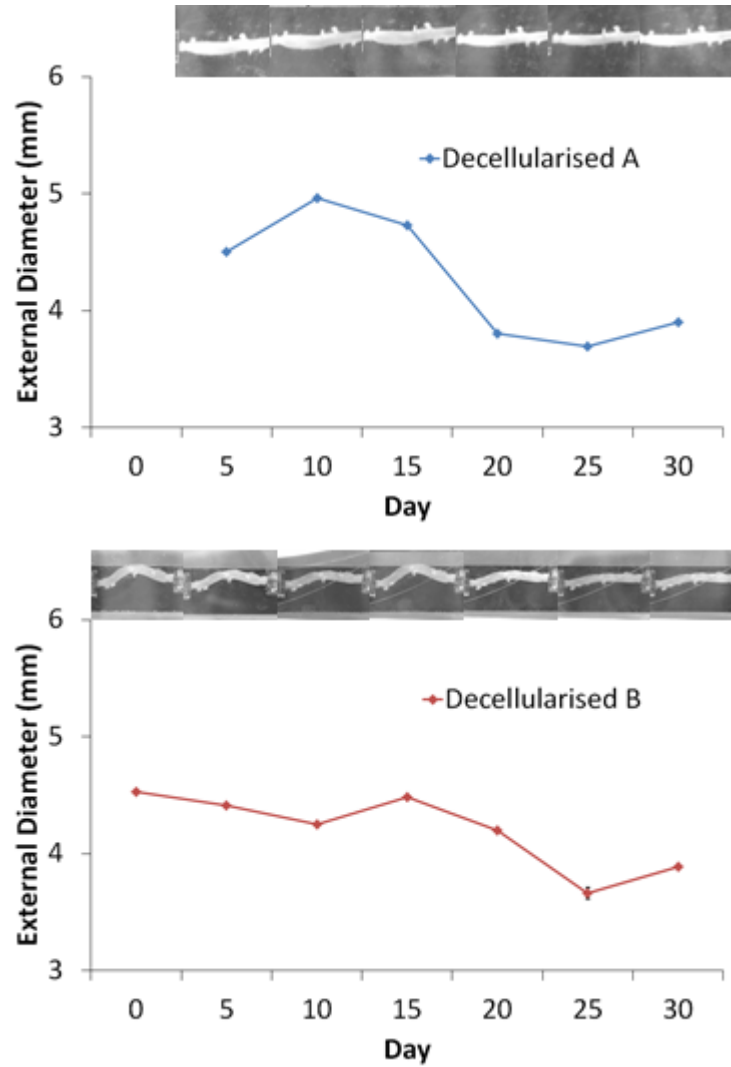


Figure 5.27: Biomechanical analysis of the effect of pulsatile flow at physiological pressures on 15-hour decellularised porcine coronary arteries. External diameter at 100 mmHg and corresponding images for each time point.

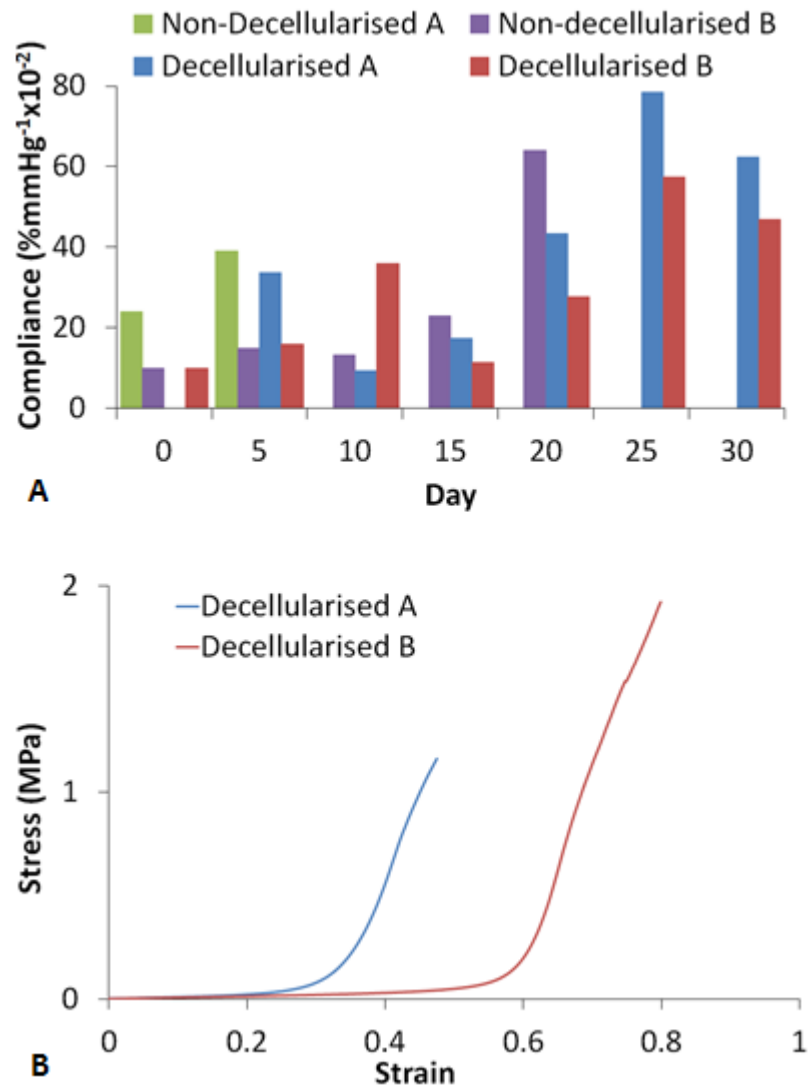


Figure 5.28: Biomechanical analysis of the effect of pulsatile flow at physiological pressures on non-decellularised and decellularised porcine coronary arteries. (A) Compliance measurement at 100 mmHg; and (B) uniaxial tensile tests of decellularised vessels after 30 days.

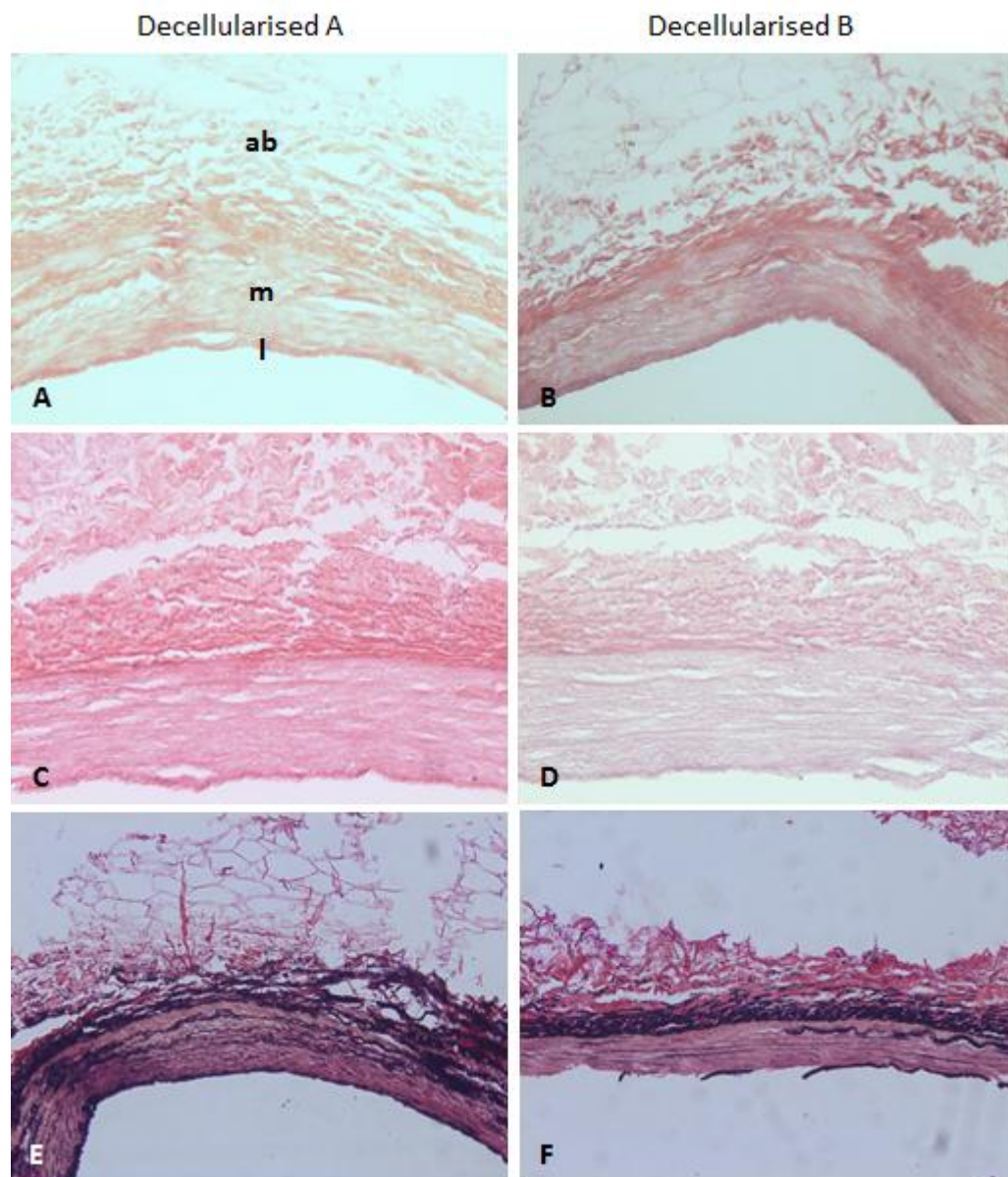


Figure 5.29: Decellularised porcine coronary arteries after 30 days of pulsatile flow at physiological pressures in a bioreactor. (A-D) H&E and (E-F) Modified Verhoeff Van Gieson Elastic staining of the arterial cross-sections. l: luminal surface, m: media, ab: abluminal surface. Magnification: (A-B, E-F) 10x, (C-D) 20x.

5.2.7 Attachment of Mesenchymal Stem Cells to Scaffold

It is clear that rMSCs adhere to the PCA scaffold after 15 days of static culture, see Figure 5.30 and Figure 5.31. A uniform distribution of cells was evident for both low and high concentrations on the abluminal surfaces, see Figure 5.30 (A, B, D, E). DAPI staining showed a low number of cells on the luminal surface mainly clustered near the lateral edges of the sections, see Figure 5.30 (C, F), similar to that found in BASMC-seeded sections (Figure 5.14). There was a minor degree of infiltration of rMSCs in the abluminal and lateral surfaces after 15 days, see Figure 5.31. However, the higher cell concentration did not increase the degree of infiltration into the scaffold. At the lower cell density a pattern of cells clustering was visible which was not apparent in the higher density sections, see Figure 5.31 (A). The intimal barrier, the internal elastic lamina, remained intact and resulted in no cell infiltration through the luminal surface.

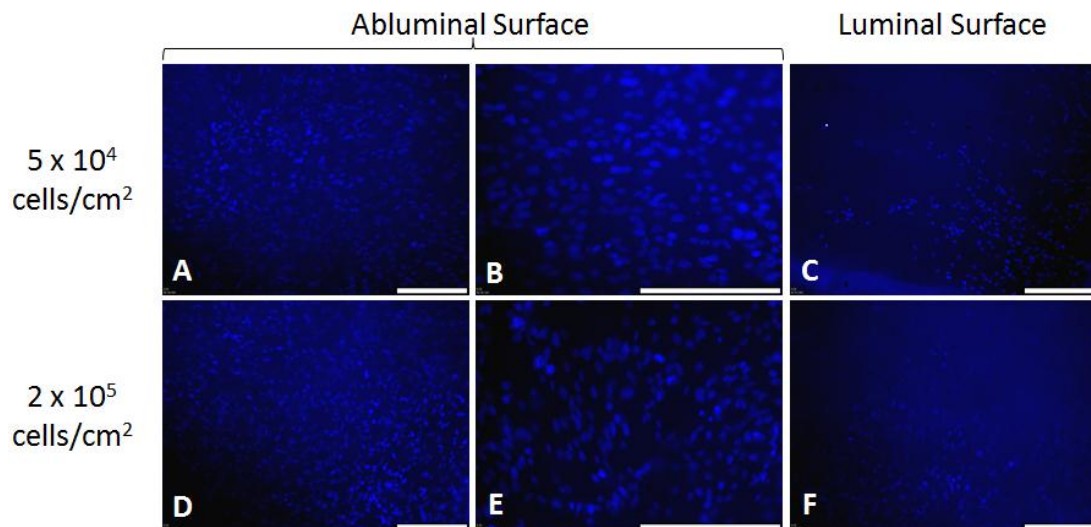


Figure 5.30: Rat mesenchymal stem cell seeding of 15 hour decellularised porcine coronary arteries. DAPI staining of the abluminal and luminal surfaces after 15 days culture at concentrations of (A-C) 5×10^4 cells/cm² and (D-F) 2×10^5 cells/cm². Scale bar: 200 μ m.

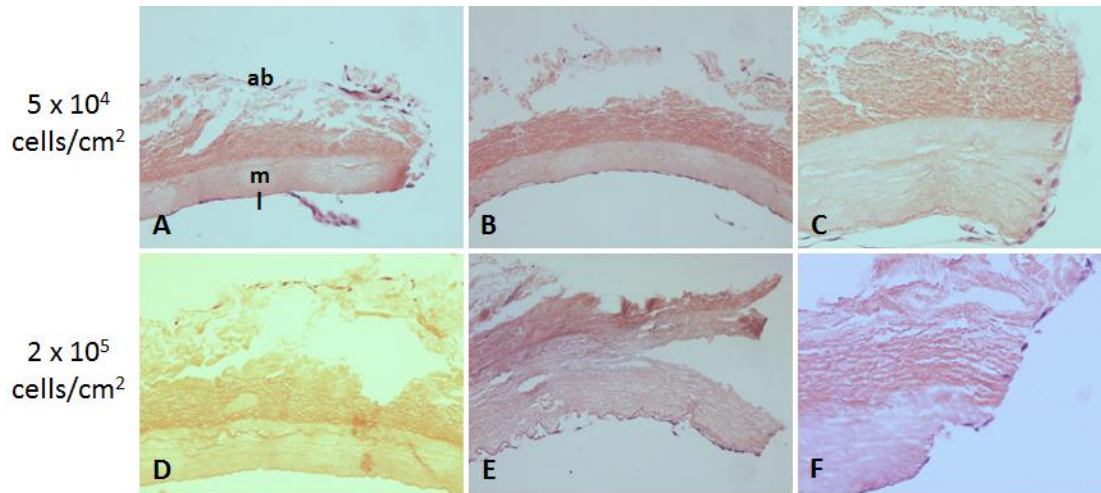


Figure 5.31: Rat mesenchymal stem cell seeding of 15 hour decellularised porcine coronary arteries. H&E staining of the cross-sections after 15 days culture at concentrations of (A-C) 5×10^4 cells/cm² and (D-F) 2×10^5 cells/cm². l: luminal surface, m: media, ab: abluminal surface. Magnification: (A-B, D-E) 10x, (C, F) 20x.

5.2.8 Attachment of Human Coronary Artery Smooth Muscle Cells to Scaffold

HCASMC were seeded to the abluminal surface of 15 hour decellularised PCA at a concentration of 2×10^4 cells/cm². DAPI staining confirmed the adherence of HCASMC to the abluminal surfaces after 1 and 15 days, see Figure 5.32. A low number of cells adhered overall, however, there was a statistically significant 3.5-fold increase in the number of cells attached to the abluminal surface after 15 days, Figure 5.33.

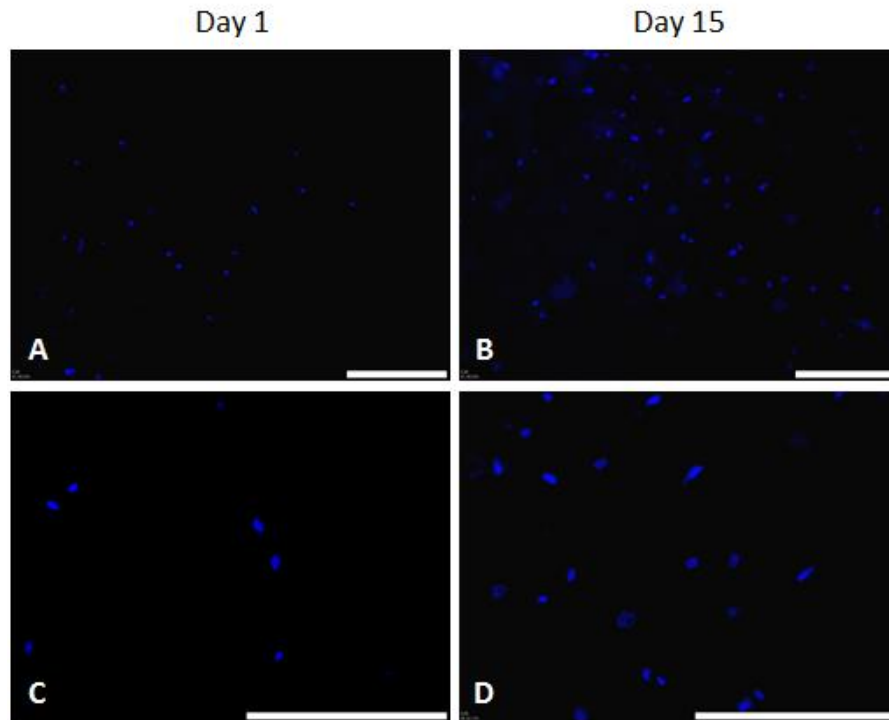


Figure 5.32: Human coronary artery smooth muscle cell attachment to the abluminal surface of 15 hour decellularised porcine coronary arteries. DAPI staining of the abluminal surface seeded at a concentration of 2×10^4 cells/cm² after 1 day and 15 days. Scale bar: 200 μ m.

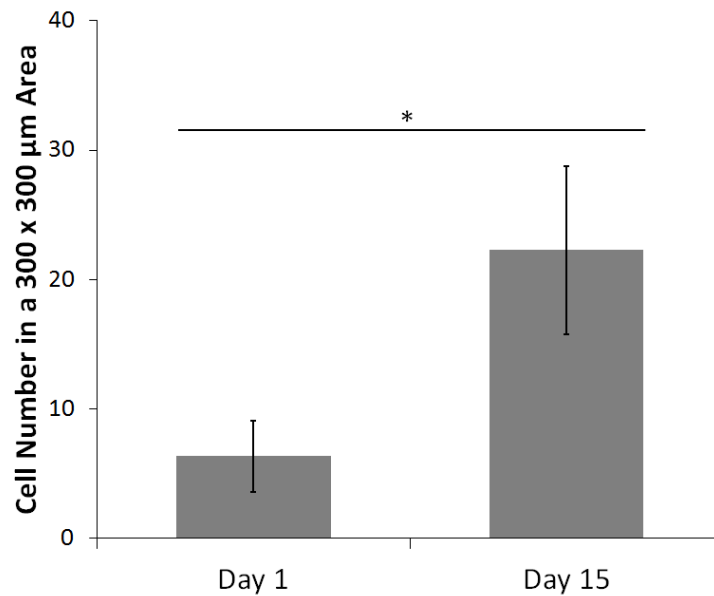


Figure 5.33: Human coronary artery smooth muscle cell count after 1 and 15 days culture on the abluminal surface of 15 hour decellularised porcine coronary arteries.

5.3 Discussion

To perform as a viable tissue engineered blood vessel scaffold, the decellularised porcine coronary artery scaffold must be capable of maintaining cells; allowing growth, infiltration and migration. This study is the first to seed cells to the natural matrix scaffold of PCA. While the tubular structure of decellularised PCA would be required for use as a TEBV, in order to determine the capability of the scaffold for repopulation, flat sections were initially seeded with cells. The cell studies performed demonstrate the suitability of the PCA scaffold for xenogeneic cell growth, attachment, and infiltration. Without the use of adhesion molecules to promote cell attachment, BASMC adhered to both the luminal and abluminal scaffold surfaces after 24 hours of static culture, see Figure 5.8. However, the internal elastic lamina proved to be a barrier to cell infiltration from the luminal surface. The exposure of vSMCs to blood flow may encourage over proliferation leading to intimal hyperplasia as demonstrated in a cyclic strain environment [128]. For a potential TEBV, the maintenance of the internal elastic lamina is a positive outcome as an intact internal elastic lamina could support endothelial cell seeding without the risk of smooth muscle cell neointimal formation. Consequently, the more loosely packed adventitial layer appeared the most favourable approach for scaffold repopulation. The composition of the natural matrix scaffold is vital as shown by Gui et al. [95]. In that study of *in vivo* repopulation of decellularised umbilical artery, the dense collagen matrix resulted in no cellular infiltration after 8 weeks implantation. Similarly, the adventitial layer of PCA scaffold is denser towards the media and may not support cellular infiltration, see Figure 5.9. Furthermore, as an additional barrier to BASMC-repopulation of the media, the external elastic lamina separates the media from the adventitia. Similar to the internal elastic lamina preventing cells moving into the media from the luminal surface, the external elastic lamina may also prove to be a barrier against SMC infiltration of the media from the abluminal surface. Consequently, the most encouraging approach for SMC repopulation of the media is from the lateral edges of the scaffold. Cells were observed infiltrating the media after 15 days, see Figure 5.9 (J, P). This is encouraging for *in vivo* scaffold repopulation as an end-to-end or end-to-side anastomosis of the scaffold to the native coronary

arteries could potentially repopulate the graft without the need for pre-population of the medial layer of the scaffold.

A further indication of cell migration was evidenced on the luminal surface of abluminally-seeded sections. The luminal surface remained flat on the base of the culture plate for the duration of culture. However, after 5 days, cells were visible clustered at the edges of the luminal surfaces, see Figure 5.14 (A-D). There were no cells visible in the central areas of the luminal surface at this stage, however, after 15 days BASMC covered all areas of the luminal surface, see Figure 5.14 (E-H). Mesenchymal stem cells seeded under the same conditions showed an equivalent pattern, see Figure 5.30; however, in these preliminary MSC experiments the phenotype was not assessed. Moreover, this behaviour is promising for in vivo repopulation of the scaffold implanted without pre-seeding.

A degree of scaffold porosity is required to enable cell infiltration of a TEBV, however, it should not compromise the structural and biomechanical properties required to perform suitably as a TEBV. An increased NaOH decellularisation time results in a scaffold with increased porosity without an adverse biomechanical effect, see Table 4.1 (pg. 78). Therefore, cell seeding studies were performed on PCA decellularised for 9, 12, and 15 hours. An increased cell number and a greater degree of infiltration, in particular in the medial layer, was demonstrated by 15 hour decellularised PCA as opposed to PCA treated for 9 hours and 12 hours, see Figure 5.12.

PCA scaffolds were pre-treated with fetal bovine serum to encourage cell infiltration; however, there was no noticeable improvement in infiltration as a result. In fact, as a consequence of perfusing the scaffold with FBS, in some instances there appeared to be a degree of damage to the luminal surface thereby fragmenting the internal elastic lamina in areas, see Figure 5.15 (E, G). This could potentially lead to cells infiltrating through the internal elastic lamina; a negative outcome for a TEBV potentially leading to thrombus and neointimal formation.

In an effort to improve scaffold repopulation, an increased cell seeding concentration was applied. However, rather than increasing cell infiltration, the cells preferentially attached to each other rather than the scaffold, see Figure 5.15 (D, F). To overcome

this behaviour, rather than seed a high concentration of cells simultaneously, cells were seeded in low numbers over a period of days. As a result, a higher total number of cells were seeded to the scaffold and there was no evidence of cell clustering as previously observed, see Figure 5.20 and Figure 5.25. While the cell numbers that infiltrate the scaffolds remain low in comparison to non-decellularised PCA this approach is promising for future in vitro scaffold repopulation studies.

Studies of other decellularised vessels have examined the ability of endothelial cells to form confluent layers on luminal surfaces. With the aid of fibronectin, Gui et al. [95] seeded human umbilical vein endothelial cells to flat segments of decellularised human umbilical artery. Liu et al. [96] seeded porcine aortic endothelial cells on flat strips of decellularised descending aorta of fetal pigs. Almost confluent layers were achieved in static culture within 5 days and 7 days, respectively. In this current study, BAEC were seeded to the luminal surface of PCA scaffold at a low concentration to observe the development of an endothelial layer with time. BAEC formed an almost confluent layer after 10 days of static culture without the use of adhesion molecules, see Figure 5.17. There was a 22-fold increase in the number of cells attached to the luminal surface at 10 days compared to 1 day. Similar to other studies, there was an even distribution of cells across the luminal surface due to the contact-inhibiting nature of EC. The ability of a TEBV scaffold to support a endothelial cells is vital as demonstrated by the thrombus formation and occlusion of decellularised vessels implanted without EC pre-seeding [95,98,114].

While it has been shown that NaOH decellularised PCA can support the attachment and infiltration of BASMC, verifying its ability to maintain xenogeneic cells, the scaffold must also support human cells to function as a TEBV. HCASMC adhered to the abluminal surface of PCA scaffold after 15 days. Additionally, the attachment and infiltration of mesenchymal stem cells was achieved, see Figure 5.30 and Figure 5.31. MSCs have demonstrated the ability to differentiate into smooth muscle and endothelial cells [82]. Autologous MSCs are more readily available for use in tissue engineering since they can be harvested from bone marrow whilst obtaining autologous vascular SMC and EC necessitates the explant of a blood vessel.

To overcome issues of lack of cell infiltration in decellularised arteries, novel techniques other than the standard method of seeding cells directly to the scaffold

surfaces, have been proposed by Soletti et al. [130] and more recently by Sheridan et al. [92]. Soletti et al. [130] accomplished more uniform scaffold cell seeding using combined vacuum pressure, centrifugal force and flow. Sheridan et al. [92] embedded micro-needles in the artery during the decellularisation procedure and subsequently used the needles to inject cells and re-seed the scaffold. This current study maintained the native architecture during both decellularisation and repopulation of the scaffold.

The removal of vascular cells by NaOH results in a less stiff, more distensible low load response and a stiffer high load response of PCA as demonstrated in Chapter 4. Therefore, uniaxial tensile tests were performed on BASMC-seeded PCA scaffolds after 30 days culture to determine if there is a corresponding alteration as a result of scaffold repopulation. Additionally, further sections were tested to establish if there is degradation of non-decellularised tissue and non-seeded decellularised scaffolds with time. In addition to providing a suitable environment for cell attachment and growth, the scaffold must have appropriate biomechanical properties to withstand physiological blood pressure upon implantation. A number of studies of repopulated decellularised scaffolds have indicated an improvement in the scaffold biomechanical properties in comparison to the bare scaffold. Schenke-Layland et al. [115] reported improved radial and circumferential tensile strength of repopulated decellularised porcine pulmonary valves after 16 days of dynamic culture, in line with the biomechanical properties of fresh valves. Recently, two studies [90,104] of the repopulation of porcine carotid arteries have shown a reversal in the alteration of biomechanical properties that occurred as a result of decellularisation. In both cases, despite the use of different decellularisation methods, the repopulated arteries exhibited reduced elasticity compared to non-seeded decellularised arteries. A similar trend is exhibited in this current study after 30 days static culture of BASMC-seeded scaffolds, see Figure 5.22 and Table 5.1. The reintroduction of smooth muscle into the medial layer of the scaffold, see Figure 5.12 (P-R), has potentially established cell-matrix interactions resulting in a reduction in distensibility, exhibited by a reduced final x-intercept value, in comparison to decellularised sections. Additionally, infiltration of cells may account for the reduced variability in the seeded decellularised stress-strain response compared to non-seeded

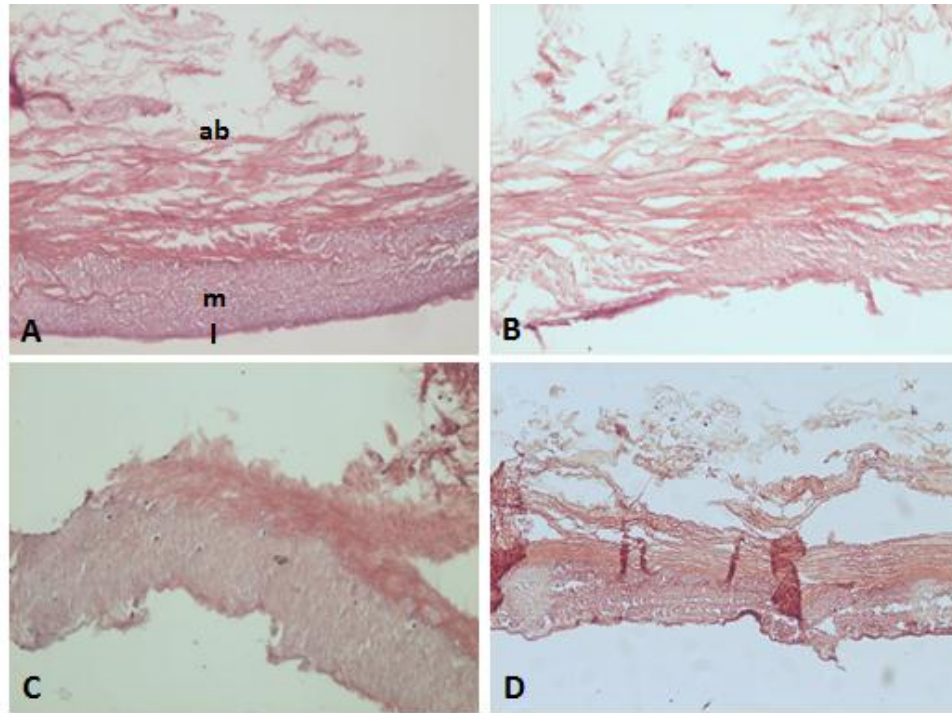


Figure 5.34: H&E sections of the four sections from artery #9 (see Figure 5.24). (A-B) Non-seeded decellularised adjacent sections and consecutive (C-D) seeded decellularised adjacent sections. l: luminal surface, m: media, ab: abluminal surface. Magnification: 10x.

decellularised sections. Conversely, non-decellularised sections have a reduced quantity of smooth muscle within the tissue due to the migration of cells from the tissue, see Figure 5.10 and Figure 5.25, and exhibit the highest degree of distensibility.

In three instances, non-seeded decellularised sections from artery #9 and non-seeded and seeded decellularised sections from artery #7, there is early recruitment of the collagen fibres resulting in a low value x-intercept compared to the remaining sections, see Figure 5.24. Analysis of the test data revealed that the gauge lengths of these particular sections after pre-conditioning were unusually high. This is demonstrated by the large standard deviations of the gauge lengths of non-seeded decellularised and seeded decellularised sections after pre-conditioning, see Table 5.1 and Figure 5.23. Histological analysis of the four sections from artery #9, see Figure 5.34, revealed that the section with the high gauge length was

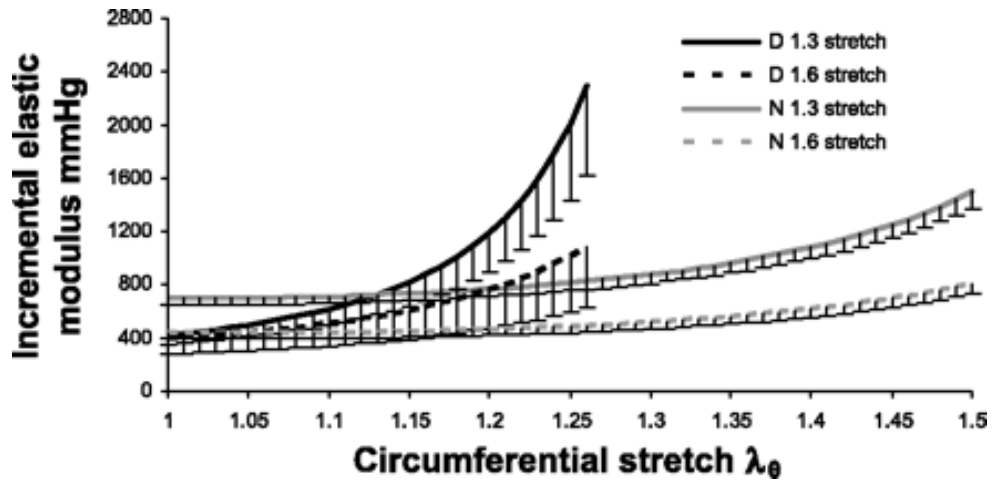


Figure 5.35: Effect of increasing pre-stretch on non-decellularised (N) and decellularised (D) porcine carotid arteries [36].

significantly more porous than the other sections, see Figure 5.34 (B). Additionally, a breakdown of the internal elastic lamina was apparent which may account for the early recruitment of collagen fibres.

SMC subjected to cyclic uniaxial strain have been shown to achieve an almost perpendicular orientation to the direction of the applied strain [131]. A Flexercell® unit designed to apply biaxial strain to cells was modified to apply a uniaxial strain to cell-seeded scaffolds. Cells were seeded to the smooth luminal surface in order to enable immunocytochemical analysis of cell alignment due to the difficulty in imaging cells seeded to the fibrous abluminal surface. Clearly, there was alignment of cells on the luminal surface of the scaffold as a result of cyclic uniaxial strain in comparison to the random orientation of static sections, see Figure 5.21. Of further interest would be the proliferation and alignment of infiltrated cells, in particular mesenchymal stem cells, under uniaxial cyclic strain. This method will enable such studies to be carried out in future following an initial culture period to enable cell infiltration.

The natural matrix scaffold of PCA demonstrated the ability to withstand physiological blood pressures and pulsatile flow for a period of 30 days without rupture or aneurysm formation. There was, however, an alteration in the biomechanical properties with time. Roy et al. [36] reported that increasing the pre-stretch of intact non-decellularised and decellularised porcine carotid arteries

decreased the compliance of the vessels, see Figure 5.35. Therefore, an inability to apply an accurate pre-stretch in this current study potentially had an impact on the resulting compliance data. A decrease in external diameter and a corresponding increase in compliance were observed after day 15 for both non-decellularised and decellularised sections, see Figure 5.28. However, the cell infiltration and the biomechanical analysis of the cell seeded scaffolds have demonstrated that BASMC cell infiltration of the PCA scaffold at 15 days can decrease vessel distensibility when compared to non-seeded decellularised sections. These observations suggest that a cell-seeded scaffold within the bioreactor environment may prevent the compliance and dimension alterations observed after 15 days.

Overall, the results presented demonstrate that vascular smooth muscle cells can infiltrate the medial and adventitial layers of the PCA scaffold from the abluminal surface and, in particular, from the lateral edges of the scaffold. Additionally, a confluent endothelial layer can be formed on the internal elastic lamina which can act as a barrier to cell infiltration from the luminal surface irrespective of decellularisation treatment time or length in culture. Xenogeneic cells, including bovine, murine, and human cells, survived for periods of between 15 to 45 days on the porcine scaffold. A combination of factors indicates that the most appropriate method to maximise scaffold repopulation is through end-to-end or end-to-side anastomosis. Seeding of a tubular length of PCA scaffold by circulating cells demonstrated that whilst an almost confluent cell layer forms on both the luminal and abluminal surfaces, there is limited potential for infiltration of the cells into the medial layer, see Figure 5.20. Successfully maintaining the PCA scaffold under pressure in a bioreactor for 30 days confirmed that the scaffold remains viable with time under pulsatile flow at physiological pressure. Therefore, the PCA scaffold has potential as a biologically and biomechanically compatible *in vivo* bypass graft, with or without pre-population with cultured cells.

Chapter 6 Final Discussion

A small-diameter tissue engineered blood vessel (TEBV) with appropriate biomechanical and structural properties is required for procedures such as coronary artery bypass grafting (CABG). Biomechanical mismatch between the native blood vessel and the bypass graft is a leading cause of bypass graft failure. In particular, compliance mismatch causes anastomotic flow instabilities potentially resulting in thrombus formation and intimal hyperplasia. In order to improve the long-term outcome of bypass grafts, it is vital to identify the appropriate biomechanical properties required of the native vessel to develop an equivalent graft. The development of compatible scaffolds from synthetic and natural materials is hindered by the complex anisotropic structure and interactions of the extracellular components within the arterial wall and has proven difficult to achieve. Decellularisation of allogeneic or xenogeneic blood vessels offers the greatest prospect of achieving a biomechanically and structurally equivalent scaffold for TEBV as the source tissue already contains all the required elements. Provided decellularisation can be achieved within an appropriate timeframe and without significant degradation of the extracellular matrix (ECM), it provides a platform to achieve the natural matrix scaffold potentially maintaining native architecture and biomechanical properties.

The small-diameter coronary arteries are difficult to harvest due to their location within the coronary and interventricular sulci of the heart and their numerous branches. However, as they are the vessels most likely to be bypassed, an investigation of their potential as TEBV scaffolds was warranted. In fact, in porcine hearts where the circulations system is almost identical to that of human hearts, lengths of coronary arteries can be successfully harvested and decellularised. As

demonstrated in the present study, the left anterior descending (LAD), right (RCA), and left circumflex (LCX) porcine coronary arteries can be excised with all branches ligated and decellularised. However, the LCX tapers more rapidly than the LAD and RCA and is unsuitable for lengths greater than 25 mm. Consequently, the LAD and RCA offer the greatest potential for use as a TEBV graft. The short-term decellularisation method successfully implemented in the current study could be applied to other blood vessels; however, it would require customisation for differing vessel dimensions and also for different vessel structures. For example, muscular arteries do not contain elastic laminae in the medial layer; therefore, if applied to similarly sized elastic arteries an increase in treatment time may be required to ensure perfusion through the elastic laminae. Furthermore, the removal of cells from the scaffold may be hindered by these additional barriers. This is demonstrated by a repopulation study where cells seeded between elastic lamina of the media were unable to infiltrate other layers [92].

Sodium hydroxide (NaOH) treatment of porcine coronary arteries (PCA) alters the biomechanical properties such that a less stiff, more distensible low load response and a stiffer high load response results, see Table 4.1 (pg. 81). The reduction in stiffness in the low load range is not statistically significant and, in fact, non-decellularised PCA tested 9 hours after excision also exhibited a reduction in the low load stiffness although to a lesser extent. Essentially, the increased distensibility can be recovered upon scaffold repopulation through cell-matrix interactions and the activation of smooth muscle tone. The increase in stiffness in the high load region, where collagen dominates the response, is the most significant alteration as a result of NaOH decellularisation. However, the normal physiological pressure range of 120/80 mmHg corresponds to 0.011/0.016 MPa, which is in the low load region of the stress-strain response. Therefore, this increase should not adversely affect the biomechanical response under normal conditions. Infiltration of BASMC into the media and adherence of other cell types to the luminal and abluminal surfaces of decellularised scaffolds indicates that cytotoxic NaOH has been completely removed from the scaffold. Moreover, 0.1M NaOH immersion for 24 hours has been shown to disrupt the collagen network and alter the stress-strain response of decellularised arteries [92]. In this current study, after 30 days of static culture, there is no further

degradation of decellularised tissue (D_{30}) demonstrated by no statistically significant difference of D_{30} in comparison to the final slope and final x-intercept of 15 hour decellularised sections (D_{15}), see Table 6.1. This further demonstrates the removal of NaOH from the scaffold and, additionally, the preservation of the elastin and collagen matrix of decellularised tissue with time. Consequently, long-term storage of decellularised PCA is viable and therefore strengthens the argument for its use as a graft. Storage and preservation of decellularised vessels would allow for patient-specific grafts to be developed; as a range of lengths, diameters and thicknesses could be available. The availability of synthetic materials as “off-the-shelf” grafts, despite the negative implications of biomechanical mismatch, was a significant advantage over natural materials; however, this outcome demonstrates that natural materials can also be stored.

Recent studies of the repopulation of elastic arteries have shown recovery of the biomechanical properties that occurred as a result of decellularisation [90,104]. This current study hypothesised that the same trend may be applicable to small-diameter muscular coronary arteries. Non-decellularised tissue cultured for 30 days (N_{30}) exhibits statistically significant less stiff, more distensible properties than non-decellularised arteries tested immediately post mortem (N_0), see Table 6.1. Essentially, N_{30} exhibits biomechanical properties comparable to decellularised tissue, D_{15} and D_{30} . The migration of smooth muscle cells from the tissue, similar to the removal of cells by decellularisation, results in a tissue with greater distensibility. Although, the effect is more pronounced in non-decellularised PCA as the distensibility of N_{30} occurs at a statistically significant higher value than D_{15} . Additionally, the decreased stiffness of the high load region illustrates a degradation of the collagen fibres. Furthermore, the increase in final stiffness of decellularised sections can therefore be linked to the decellularisation protocol rather than purely the removal of vascular cells. The alteration of N_{30} in comparison to N_0 also suggests that the alteration in biomechanical properties observed in non-decellularised tissue frozen for extended periods [23,93] is a result of damage to the vascular cells in combination with a deterioration of the extracellular matrix. A limitation of this work includes the use of a low resolution imaging technique which cannot provide conclusive data on disruption of collagen or elastin fibres following decellularisation

Table 6.1: Uniaxial tensile test data comparison of sections tested within 24 hours of slaughter and after 30 days culture. Non-decellularised (N_0) and 15 hour decellularised (D_{15}) sections were tested within 24 hours of slaughter. Non-decellularised (N_{30}), 15 hour non-seeded decellularised (D_{30}) and 15 hour seeded decellularised sections (S_{30}) were tested after 30 days of culture.

	Final Slope, m_f (MPa)	Final X-Intercept
$N_0, n=25$	7.35 ± 2.71	0.40 ± 0.08
$N_{30}, n=6$	4.23 ± 1.23^1	0.58 ± 0.04^1
$D_{15}, n=19$	8.08 ± 4.45	$0.48 \pm 0.07^{1,2}$
$D_{30}, n=10$	11.10 ± 6.38^2	0.50 ± 0.23
$S_{30}, n=10$	$10.34 \pm 2.56^{1,2}$	0.42 ± 0.12^2
Significance, ¹ $p < 0.05$ from non-decellularised (N_0) sections		
² $p < 0.05$ from non-decellularised (N_{30}) sections		

with NaOH. It should be noted however that the degradation of collagen cross-links is highly unlikely given that the final slope of the stress-strain curve, where collagen predominantly bears the load, is stiffer for the decellularised arteries when compared to non-decellularised tissue. Of greatest significance is the effect of repopulation of the scaffold on the stress-strain response. Similar to D_{15} and D_{30} , the final slope of BASMC-seeded decellularised PCA (S_{30}) after 30 days remains stiffer than non-decellularised tissue. However, the final x-intercept is comparable to non-decellularised (N_0) sections indicating a recovery of the alteration that occurred as a result of decellularisation. Restoring the native biomechanical properties is important for compliance matching of a decellularised bypass graft to the native vessel. The compliance of blood vessels and blood vessel grafts at 100 mmHg are demonstrated in Figure 6.1. There is a 4-fold increase in the compliance of

non-decellularised and decellularised PCA in comparison to other vessels and grafts. Although the graph demonstrates the distensibility of vessels and grafts within the normal physiological pressure range, the compliance over a range of pressures is important. Dacron exhibits a higher compliance measure than human saphenous vein at 100 mmHg, however, over a range of pressures it is in fact the stiffest material demonstrating minimal change in diameter.

Another limitation for the commercial viability of a TEBV scaffold production is the time it takes to repopulate the scaffold. After 45 days of static culture, a significant area of the scaffold remains acellular, see Figure 5.12 (pg. 117). In addition to providing a suitable environment for cell attachment and growth, the scaffold must have appropriate biomechanical properties to withstand physiological blood pressure upon implantation. However, PCA scaffold has demonstrated the ability to withstand physiological pressures and pulsatile flow in a bioreactor and maintains the non-linear stress-strain response, see Figure 5.27 (pg. 134) and Figure 5.28 (pg. 135). Additionally, the internal elastic lamina remains intact and is a suitable blood-contacting surface [76]. Consequently, PCA scaffold could be implanted in vivo without pre-seeding and cells in the adjacent tissue and circulating cells could repopulate the scaffold with time. This is supported by the repopulation of the medial layer of the scaffold by infiltration of the lateral edges and migration of cells across the luminal and abluminal surfaces, see Figure 5.12 (pg.117) and Figure 5.14 (pg. 119). Alternatively, pre-seeding of the scaffold with autologous mesenchymal stem cells (MSCs) offers great potential due to their ability to differentiate into smooth muscle cells in a cyclic uniaxial strain environment [82]. Attachment and infiltration of MSCs to the PCA scaffold was achieved, see Figure 5.31 (pg. 138). In combination with the uniaxial strain environment of the Flexercell, using the set up described in this current work, see Figure 5.2 (pg. 104), or the 3D environment of the bioreactor the repopulation of the media and the generation of ECM proteins could be enhanced.

Porcine coronary arteries are three times more elastic than human arteries [13]. Decellularisation of PCA results in a further increase in vessel distensibility. Interestingly, non-seeded decellularised (D_{30}) and seeded decellularised (S_{30}) uniaxial tensile sections which demonstrated early recruitment of collagen fibres

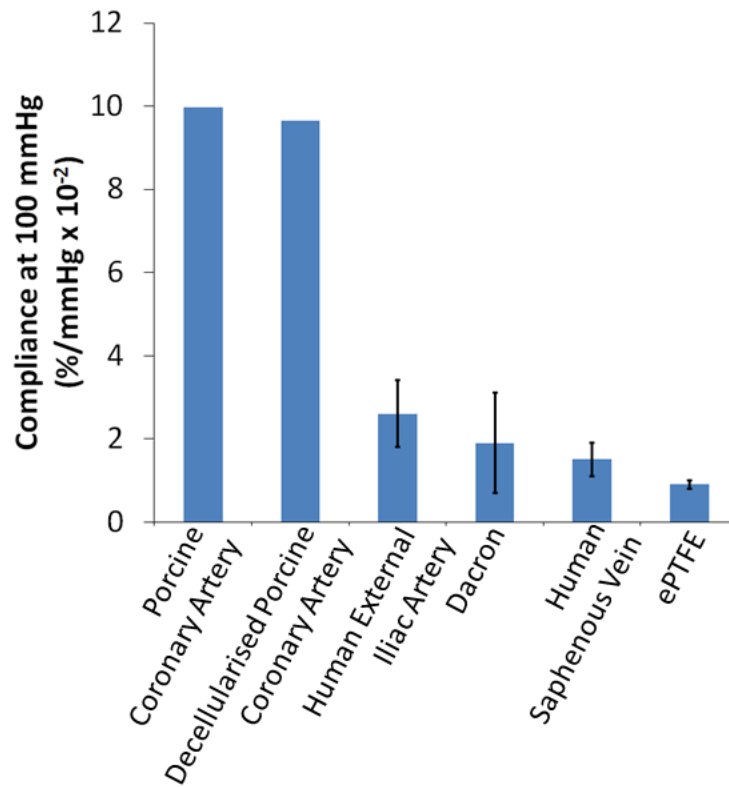


Figure 6.1: Compliance at 100 mmHg for non-decellularised and decellularised porcine coronary arteries in comparison to other blood vessels and grafts (human external iliac artery, Dacron, human saphenous vein, and ePTFE data adapted from [122]).

exhibited elastic behaviour similar to that of human coronary arteries. This can be attributed to a breakdown of the internal elastic lamina. A main objective of this work, which was ultimately achieved, was to maintain the native architecture of coronary arteries throughout decellularisation and repopulation. Unlike other studies, where adventitia was removed to improve either decellularisation or repopulation [65,91,92,99] or the media was disrupted to aid repopulation [92], in this current study the media and adventitia were maintained in their native configuration. However, controlled degradation of the internal elastic lamina, for example through uniform perforations of the surface, could reduce the distensibility of porcine tissue to bring it in line with human arteries. However, this would require careful consideration as the positive outcomes of maintaining the internal elastic lamina could potentially be lost.

The insights gained into coronary arteries and the corresponding natural matrix scaffold can be used to guide the development of other synthetic or natural materials for small-diameter TEBV scaffolds such as bacterial cellulose [79]. The properties of decellularised PCA and cell-seeded decellularised PCA are compared to human coronary arteries, current bypass grafts, and other small-diameter tissue engineered blood vessels in Table 6.2. Based on the results of this study, prior to cell seeding a scaffold with increased distensibility at low loads, decreased thickness, and increased permeability in comparison to the native artery may prove to be the optimum environment for cell growth and infiltration. Furthermore, upon cell seeding, the biomechanical properties of the native vessel can be recovered resulting in a biomechanically compatible TEBV in the long-term, see Table 6.1.

Table 6.2: Comparison of human coronary arteries to blood vessels, synthetic grafts and tissue engineered blood vessels for use as small-diameter bypass grafts. HCA: human coronary artery, HSV: human saphenous vein, HLITA: human left internal thoracic artery, ePTFE: expanded polytetrafluoroethylene, TEbV: tissue engineered blood vessel, PCA: porcine coronary artery, n/a: not available.

	HCA	HSV	HLITA	Dacron	ePTFE	Niklason TEbV	L'Heureux TEbV	PCA Scaffold	Seeded PCA Scaffold
Source	Native vessel	Autograft	Autograft	Synthetic graft	Synthetic graft	Cell-seeded biodegradable PGA scaffold	Sheet-based tissue engineering	Bare decellularised xenograft	Cell-seeded decellularised xenograft
Preparation Time	-	Upon explantation	Upon explantation	Off-the- shelf	Off-the- shelf	8.5 weeks [117]	~28 weeks [86]	24 hrs	n/a
Length (mm)	20 – 40 [13]	311±101 [119]	180.8±0.07 [118]	unlimited	unlimited	n/a	170 – 210 [66]	20 – 40	20 – 40
Wall Thickness (mm)	0.87±0.23 [20] 0.89±0.2 1 [13]	0.40±0.12 [119] 0.79±0.02 [120]	0.70±0.07 [13]	n/a	n/a	0.38±0.04 [71]	0.2 – 0.6 [66,87]	0.46±0.11	0.41±0.07
Diameter (mm)	4.5±0.3 ¹ [20] 3.54±0.5 1 ¹ [13]	4.2±0.6 ^{1,2} [119]	3.05±0.5 ¹ [118] 3.28±0.06 ¹ [13]	< 6 ³	< 6 ³	3.1 ³ [117]	1.5 – 6.6 ³ [66,87]	3.16±0.55 ¹	n/a
Opening Angle (°)	n/a	~120 ⁴ [120]	n/a	n/a	n/a	n/a	n/a	18.59±27.52	n/a

¹External diameter

²Fully distended measurement

³Internal diameter

⁴Estimated from published graph, values not published

	HCA	HSV	HLITA	Dacron	ePTFE	Niklason TEBV	L'Heureux TEBV	PCA Scaffold	Seeded PCA Scaffold
Burst Pressure (mmHg)	Up to 200 ⁵ [13]	1599±877 [66]	3196±1264 [66]	n/a	1323±383 [121]	2150±700 [117]	3399 – 3688 ⁶ [66,87]	Min. of 200 ⁵ . Flat segment 1500	Min. of 120 ⁶
Compliance (%mmHg ⁻¹ ×10 ⁻²)	n/a	5.0±0.7 [122]	11.5±3.9 [66]	1.8±1.2 [122]	1.2±0.3 [122]	<10 in physiological range [117]	3.4±1.6 [66]	9.64	n/a
Ultimate Tensile Strength (MPa)	1.30±0.69 ^{7,8} 0.42±0.19 ^{7,9} 0.39±0.14 ^{7,10} [20]	2.2±0.2 [123]	n/a	n/a	14.03±0.72 ⁷	1.2 ¹¹	1545±526 gram- force ¹² [66]	1.17±0.30 ¹³	1.08±0.41 ¹³
Maximum Incremental Modulus (MPa)	n/a	n/a	n/a	n/a	n/a	66 ¹¹ [117]	n/a	8.08±4.45 ⁷	10.34±2.56 ⁷

⁵Established during inflation test.

⁶Established during bioreactor test.

⁷Longitudinal direction

⁸Adventitia

⁹Media

¹⁰Intima

¹¹Diameter-dependent

¹²Ring segment

¹³Tested after 30 days incubation

Chapter 7 Conclusions and Future Work

7.1 Main Findings

The aim of this study was to establish a short-term decellularisation method for porcine coronary arteries (PCA) and to determine the biomechanical properties and cell repopulation viability of this natural matrix scaffold to assess its suitability as a tissue engineered blood vessel (TEBV) scaffold. In addition, the data obtained can be used to inform the development of alternative TEBVs and TEBV scaffolds for use in small-diameter bypass application. The following key contributions have been established in this study:

- The removal of the vascular cells from porcine coronary arteries has been achieved through a short-term decellularisation method. This study presents a protocol which results in the successful decellularisation of fresh tubular sections of PCA within 9 hours of excision. Given the short time for decellularisation, natural degradation of the arterial tissue is minimised during the process. Despite widespread decellularisation of other blood vessels, this study is the first to successfully obtain the natural matrix scaffold of the muscular coronary artery.
- The characterisation of the biomechanical properties of PCA scaffolds in comparison to non-decellularised PCA provides an insight into the alteration that occurs as a result of the removal of vascular cells. The resulting scaffold maintains a non-linear response to stress and the ability to withstand

pressures in excess of the physiological blood pressures. The excellent biomechanical properties of the porcine natural matrix scaffold, and the short timeframe within which this scaffold was achieved, establishes the potential of this natural matrix scaffold for use as a vascular tissue engineering scaffold. Furthermore, the scaffold exhibits a less stiff, more distensible low load and a stiffer high load response in comparison to the native vessel. This provides a suitable benchmark for the development of other vascular grafts for CABG and similar applications.

- The PCA scaffold is a suitable environment for xenogeneic cell growth, infiltration, and migration. Scaffold repopulation via the lateral and abluminal surfaces, the maintenance of the internal elastic lamina as a barrier to smooth muscle cell infiltration, and the short-term formation of an almost confluent layer of endothelial cells all provide evidence of the suitability of this scaffold for use as an in vivo vascular graft.
- The repopulation of the PCA scaffold reversed the changes in biomechanical properties that were due to removal of vascular cells, restoring the native characteristics. Additionally, the preservation potential of the decellularised matrix, as demonstrated from the longer-term tests, shows that tissue could be stored with the biomechanical properties preserved. This could enable a bank of tissue to be generated from which a scaffold could be chosen to match the compliance of patient-specific host vessels.
- The PCA scaffold has potential for use simply as a vascular graft given that its acellular nature does not initiate an immune response, it retains its structural strength and it has biomechanical properties very close to that of the native artery whilst also maintaining sufficient burst strength.

7.2 Future Work

Following on from the significant contributions outlined above, there is wide ranging potential for further development and analysis of the porcine coronary artery natural matrix scaffold as a tissue engineered blood vessel:

- Assessment of an in vitro anastomosis of non-decellularised and decellularised vessels in the bioreactor to validate the ability to repopulate the scaffold through the lateral edges, followed by in vivo animal models of pre-seeded and bare scaffolds.
- Adaptation of PCA scaffolds to achieve equivalent elasticity to human coronary arteries by controlled degradation of the internal elastic lamina. A further alignment of the biomechanical properties of PCA scaffold to human coronary arteries would enhance the compliance matching capabilities of the scaffold.
- Further analysis of human cell seeding of the PCA scaffold and, in particular of human mesenchymal stem cells. The ability of MSCs to be differentiated into vascular smooth muscle cells (vSMCs) and endothelial cell (ECs) using cyclic strain and shear stress, respectively, within the 3D scaffold environment could establish a suitable autologous cell source for TEBV development.
- Further exploration of the effect of the application of a cyclic strain environment on cell-seeded decellularised scaffolds, in particular cell alignment and extracellular matrix production in the media, and the corresponding impact on the biomechanical properties. The Flexercell® system could be used to analyse the uniaxial strain environment and the bioreactor could establish the effect of a biaxial strain environment. Furthermore, co-culture of smooth muscle cells and endothelial cells can be accomplished in the bioreactor. This work could develop an optimum loading regime to achieve controlled alteration of the natural matrix scaffold by cell growth and extracellular matrix production. In this way, the decellularised constructs could be tuned to have matching biomechanical properties for a range of different host vessels.

These findings would more conclusively establish the potential of the PCA scaffolds for use as vascular grafts or replacement blood vessels in humans.

In addition to its potential for tissue engineering applications, the bare and cell-seeded PCA scaffold could be established as a model system to investigate

pathological conditions such as the development of atherosclerosis, intimal hyperplasia, and aneurysms. Physiological versus pathological strain environments can be simulated in the Flexercell® and bioreactor environments [128]. Additionally, controlled degeneration of the scaffold, such as localised degradation of tissue simulating an aneurysm or stent deployment to initiate in-stent restenosis, could also be applied. This could further establish the role of the extracellular matrix and vascular cells, in particular circulating endothelial progenitor cells and resident vSMCs and ECs, in the pathogenesis of vascular disease.

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Appendix A

Histological Protocol

Fixing

10% formalin (4% formaldehyde) at 4 °C	12 hours
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For storage after fixing

70% ethanol	Up to 3 days
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Dehydration, Clearing and Paraffin Embedding

Volume of liquid should be 10 – 20 times that of the tissue

Dehydration

1. 70% ethanol	45 minutes
2. 95% ethanol	45 minutes
3. 100% ethanol	45 minutes
4. 100% ethanol	45 minutes

Clearing

5. Xylene	60 minutes
6. Xylene	60 minutes

Paraffin Embedding

7. Paraffin	60 minutes
8. Paraffin	60 minutes
9. Embed	

Storage

Can be frozen at -20 °C indefinitely

Microtome

Float 5 µm sections in distilled water at 37 °C before mounting on slide. Allow slide to dry out overnight before staining, otherwise section will be removed from slide once placed in xylene as water is immiscible with xylene. For quick drying the slides can be placed in the oven at 65 °C for 1 – 2 hours. Slides should be cooled to room temperature before beginning staining procedure.

Deparaffinisation and Rehydration

After each step remove excess fluid by shaking and blotting slides.

Liquids can be used repeatedly and should be renewed when traces of sludge (precipitated wax) appears in the 100% ethanol.

1. Xylene	Agitate (3 or 4 times) (Stationary	2 – 3 minutes 5 minutes)
2. Xylene	Agitate	1 minute
3. 100% ethanol	Agitate	1.5 – 2.5 minutes
4. 95% ethanol	Agitate	1 minute
5. 70% ethanol	Agitate	≥ 1 minute

Slides can be left for several hours or days in 70 % ethanol, preventing fungi and bacterial growth. This will not remove sections from the slides or make the sections brittle.

6. Distilled Water	Agitate (Stationary	30 seconds 2 – 3 minutes)
7. Distilled Water	Agitate	Dip

Hematoxylin and Eosin Staining

Hematoxylin should be protected from light.

Hematoxylin can be reused many times.

Eosin can be used repeatedly; however moulds can grow but can be removed by filtration.

- | | |
|---|-----------------|
| 1. Hematoxylin | 10 – 15 minutes |
| As Hematoxylin is diluted with repeated use, the time required to stain the tissue increases with used Hematoxylin. | |
| 2. Running Tap Water | 10 minutes |
| 3. 0.1% hydrochloric acid | Dip 3 times |
| 4. Tap Water | Dip 3 - 4 times |
| 5. Scott's Tap Water Substitute | Dip 3 times |
| 6. Tap Water | Dip 3 – 4 times |
| 7. Eosin | 30s – 3 minutes |

Dehydration, Clearing and Mounting

Dehydration

- | | |
|-----------------|------------|
| 1. 95% ethanol | 30 seconds |
| 2. 100% ethanol | 30 seconds |
| 3. 100% ethanol | 30 seconds |

Clearing

- | | |
|-----------------------------------|------------|
| 4. Xylene | 30 seconds |
| 5. Xylene | 30 seconds |
| 6. Mounting media and cover slide | |