Fundamental Aspects of Tissue Engineering in order to Construct a Functional Aortic Heart Valve

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This thesis is dedicated to my wife Ria and daughters Seema and Kirti.
Live as if you were to die tomorrow. Learn as if you were to live forever.

Mahatma Gandhi
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The cardiac surgeon did not get to the summit by himself.

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Introduction

1. Aim of the study

Current bioprosthetic valves are far from ideal. The reasons for this lie on one hand with the complexity of the native aortic valve and on the other with inherent limitations of bioprosthetic valve themselves. A tissue engineered aortic valve involves the development of a vital construct which mimics the native aortic valve and which can replace diseased aortic valves without the limitations of currently available prosthetic valves. Tissue engineering was defined by The World Technology Panel Report [1] as: ‘The application of principles and methods of engineering and life sciences to obtain a fundamental understanding of structure-function relationships in novel and pathological mammalian tissues and the development of biological substitutes to restore, maintain or improve tissue function’.

When finally manufactured a tissue engineered aortic valve will be required to replicate most, if not all, of the biological functions of a normal aortic valve and the normal valve’s morphodynamic behaviour [2] in relation to the heart.

Although tissue engineering is being currently applied to neural tissue, skin, bone, cartilage and liver among others [3], to date, the successful development of a functional tissue engineered aortic valve has not been achieved. Nevertheless, a tissue-engineered valve is a promising concept that can revolutionize the treatment of valvular heart disease with global impact. At present several approaches to tissue valve engineering are evolving and include the use of synthetic or decellularized allogenic or xenogenic valve matrices followed by attempts to repopulate the matrices with appropriate autologous cells, either in vitro or in vivo after implantation [4,5]. In these approaches several critical issues need to be addressed and they include the choice of matrices, cell type, whether to repopulate in vitro or rely on in vivo repopulation as well as the performance of such constructs in a large animal model before clinical use as is required by controlling agencies such as the Food and Drug Administration (USA) and the International Standards Organization (ISO 5840).

This study addresses some fundamental aspects in the development of a functional tissue engineered aortic valve using xenogenic scaffolds and fibroblastic mesenchymal cells.

Investigators in this field are faced with a major problem. No tissue engineered valve has yet been successfully developed and the exact requirements of such a prosthesis are difficult to specify, a task made all the more difficult, since the biology and bio-
mechanics of native aortic valves have only recently begun to be defined [6-9]. In fact, surrogate endpoints such as hemodynamic performance; calcification and capacity to grow have yet to be established. Much of the investigations in this study form part of this new research domain and cannot be situated in a huge realm of previous research. What is known is that the native aortic valve structure and function is complex as described below and that they succumb to disease. Further, it is known that current prosthetic valves do not match the native valve’s performance due to inherent limitations. To illustrate the complex challenges to aortic valve tissue engineering and the approaches that have been adopted to meet them in this study, a description of the complex nature of the aortic valve becomes obligatory.

2. The Challenges

2.1 The Complex Structure Function Relationships of the Aortic Valve.

2.1.1 The Heart and its Valves

A healthy human heart weighs approximately 200 to 400 grams, pumps approximately 5 to 6 litres of blood per minute when beating at a rate of 70 beats /minute and ensures a continuous and adequate supply of oxygen to the vital organs. In fact, in a single day such a heart would beat more than 100,000 times, pump more than 8,000 litres of blood and is capable of increasing its output three to four times during exercise. Blood is pumped through the large vessels connected to the heart and the heart’s valves serve to direct this flow in one direction by not allowing back flow or regurgitation. Blood returns from the body to the right atrium of the heart via the vena cava and passes through the tricuspid valve to the right ventricle. It then goes through the pulmonary valve to the lungs, where it is oxygenated and returned to the left atrium via the pulmonary veins. It then passes through the mitral valve to the left ventricle from where it goes through the aortic valve to the aorta and the rest of the body (Fig 1).

The cardiac cycle can be divided into two stages. The first is diastole during which the ventricles briefly relax before filling with blood. The second stage is systole, which represents the period of contraction and ejection of blood from the ventricles (Fig 1).

The aortic valve as such is subjected to cyclical systolic pressures in preventing backward flow into the left ventricle. The pressure changes during the cardiac cycle are illustrated in Figure 2, in which seven phases are indicated [10]. During the first
phase the atria contract (Phase 1), ejecting blood into the ventricles through the tricuspid and mitral valves (A-V valves). The subsequent increase in ventricular pressure closes the A-V valves. At the beginning of systole, ventricular isovolumetric contraction occurs (Phase 2) with a rise in ventricular pressure, which results in the opening of the aortic and pulmonary valves and the ejection of blood into the corresponding vessels (Phase 3). The aortic pressure rises above that of the emptying ventricles with closure of the aortic valve. Following the rapid ejection, both the pressure in the ventricles as well as in the aorta is reduced (Phase 4). The ventricles then isovolumetrically relax (Phase 5) and end the systolic phase after approximately 0.35 seconds. The diastolic stage follows the end of systole, with rapid filling of the ventricles (Phase 6) once the ventricular pressure falls below the atrial pressure with opening of the A-V valves. During this period, the flow of blood from the aorta to

Fig. 1. Illustration of the anatomy of the heart and its systolic and diastolic functions. Reproduced with permission. (Image copyright Texas Heart Institute, www.texasheartinstitute.org).
the peripheral circulation continues, and the aortic pressure gradually decreases. The rapid ventricular filling is followed by a slower, reduced ventricular filling phase (Phase 7).

2.1.2 The Aortic Valve Structure and Function.

The aortic valve opens and closes approximately 100,000 times per day and approximately 3 billion times in its life span [11], during which the high complexity of its structure-function relationship, guarantees optimal hemodynamic function and remarkable durability.

Figure 3 illustrates the anatomy of the normal aortic valve and its relations. Each valve is comprised of three semilunar leaflets referred to as cusps and which are inserted into the aortic wall in a semilunar fashion and in fact separates hemodynamically the aorta from the left ventricle. The aorta bulges behind the attachment

![Diagram of the Cardiac Cycle](image-url)

**Fig. 2. The Cardiac Cycle- events occurring during the cardiac cycle (Klabunde, 2004) [10].**
of each leaflet-giving rise to the aortic sinuses, which are expanded portions of the aortic wall. Distally, the aortic valve is continuous with the ascending aorta and proximally it is attached to the left ventricle. The surface of the leaflet facing the aorta is called the aortic surface and that facing the ventricle, the ventricular surface. Each leaflet has an average thickness of 0.6mm, which varies considerably over the surface [12] and is named according to its adjacent sinus. The ostia of the left and right coronary arteries open into the right and left coronary sinuses respectively. The third sinus has no coronary artery opening and is referred as the non coronary sinus. Accordingly, the three semilunar leaflets are referred to as the right, left and non-coronary leaflets respectively [11].

![Fig. 3. Anatomy of the aortic valve](image)

The morphological design of the aortic valve results in hemodynamic flow characteristics that are coupled to cardiac contraction in morphodynamic behavior. During systole the commissures, points of attachment of adjacent leaflets pull outward causing the leaflets to open. Simultaneously, the base of the valve moves inwards causing a decrease in base perimeter. During diastole, vortices developed in the sinuses, along with an inward movement of the commissures and outward movement of the base close the valve [14].

The aortic leaflets do not passively open and close during the cardiac cycle. During their movements the leaflets undergo repetitive changes in conformation and
dimension, varying their surface area by as much as 50% to ensure closure while withstanding the cyclical pressures. This extraordinary function is still not fully understood and results from complex actions and interactions of the elements comprising the leaflet [15,16].

Each valve leaflet is made up predominantly of structural (collagen and elastin) and cellular (Interstitial cells with fibroblast and smooth muscle characteristics, endothelial cells) elements that organize to form three distinct layers (Fig.4); (a) the ventricularis, (b) the spongiosa and (c) the fibrosa. The micro architecture is such that the layers display structural and non-structural regional specialization giving the valve anisotropic qualities whereby its properties are not the same in all directions [17]. Collagen fibers in the fibrosa are compact and arranged both longitudinally and radially, with the radial fibers being more corrugated (crimped) than longitudinal ones. The collagen present in the leaflets consists of collagen types I, III and V [18]. The ventricularis has less dense, longitudinally arranged, corrugated collagen and radial elastin fibers. Elastin is considerably less stiff than collagen and its coiled hydrophobic structure allows its molecules to slide over each other and provide stretch to maintain structural integrity and to provide recoil [19]. The spongiosa
contains loosely arranged collagen and abundant proteoglycan [17]. The proteoglycan content of aortic leaflets consists mainly of Dermatan and Chondroitin sulfate, and their water retaining properties allows for the dampening of mechanical forces developed during valvular function and maintaining viscoelastic properties of the tissue [20-23]. This micro architecture and its associated micro mechanics impart specific qualities to the natural valve; (a) fiber rearrangement and remodeling allow the leaflet to be soft and pliable as they open during systole but stiff and extended when closed to withstand backward pressure an prevent leaks into the left ventricle. Closure is facilitated by increased surface area accomplished by the flattening of corrugations in radial collagen fibers and elastin stretch while stiffness is generated by longitudinal fibers that flattening out by losing their corrugations [24] (b) there is differential movement of the leaflet layers, which slide and increase surface area, (c) forces generated by differential movement [25] and closure of the leaflets is absorbed by the spongiosa and transferred to the elastic aorta, thereby resulting in a minimum of force on the closed leaflet, (d) the fibroblasts which constitute a heterogeneous mesenchymal cell population with predominantly the myofibroblastic phenotype communicate with each other by an as yet not understood mechanism and continuously replenish and remodel the structural elements as they become damaged from repetitive function [24].

Moreover the movement of the valve has been shown to precede the movement of blood, and thus to occur before the hemodynamic events [26]. It is also known that valve tissue is innervated by sympathetic, parasympathetic and sensory nerve fibers and those different parts of the valve can react to circulating vasoactive products [27]. Regional specification in gene expression by the cells of the valve is believed to be responsible for these observations [28].

2.2 Heart Valve Disease.

The United Nations population report projects an increase in the world population between 2005 and 2050 from 6.4 to 8.9 billion inhabitants or more depending on world fertility pattern (Fig.6).

Valvular heart disease is a serious clinical condition resulting from the dysfunction of one or more of the heart’s valve. The incidence and prevalence of valve disease is increasing worldwide due to the world population’s increasing age (Fig. 7) and the world’s failure to address the problem of rheumatic cardiac disease in developing countries and in indigenous populations in the developed world [29] (Fig. 8). The third world will have the largest increase in world population [30] and due to limited resources not all patients requiring heart valve replacement will even have access to
this treatment. Strategies to address this global problem [31] including development of an affordable and durable prosthetic valve could have significant impact. At present, in the USA alone, approximately five million patients are diagnosed with valvular heart disease each year and according to the American Heart Association 2003 Heart and Stroke Statistical update, valvular heart disease is responsible for almost 20,000 deaths annually and is a contributing factor in almost 42,000 [32]. Aortic valve disease alone accounts for 63% of deaths and the mitral valve (14%), pulmonary (0.06%), tricuspid (0.01%) or combination accounting for the rest. More than 100,000 US patients need replacement of their dysfunctional valves with a prosthetic valve each year and more than 300,000 prosthetic valves are sold worldwide annually [33].

Disease of the aortic valve can result in stenosis, regurgitation or a combination of both and leads to pressure overloading of the heart due to a restricted valve opening or volume overloading due to inadequate valve closure. In the USA, valvular aortic stenosis and regurgitation affect about five out of every 10,000 people [34].
Fig. 7. Life expectancy at birth for the world and major areas, 1950-2050.

Fig. 8. Population dynamics by development groups, 1950-2050.
The prevalence of aortic stenosis increases with age showing an occurrence of sclerosis in 26% of adults above 65 years of age and stenosis in 4% of adults above 84 years of age [35-37]. Age related aortic degenerative aortic stenosis is the most common cause of isolated aortic valve disease in adults. It is typified by subendothelial infiltration by proteins and lipids and a progressive stiffening of the leaflet due to calcification [38-40]. Native aortic valve cusps are visibly opaque after the age of 20 years [41] and their thickness increases significantly with age as a result of thickening of the fibrosa and ventricularis layers [38]. Lipid accumulation with formation of atheromas is usually observed after the age of 30 years [43]. With aging, calcific deposits have been reported to occur in more than 20% of individuals older than 75 years [44] and are usually found within lipid accumulations deep in the fibrosa near to the spongiosa layer. Ultra-structurally, this calcification is associated with debris derived from degenerated cells [45] such that native aortic valve calcification is thought to be a degenerative change. Moreover, with age, the anatomy of the aortic valve changes, with progressive stretching, fibrosis and lipid accumulation as well as an increase in valvular diameter [46,47]. It has been suggested that subtle abnormal changes in cusp shape and size, which result in increased hemodynamic stresses, may contribute to the degenerative process leading to aortic stenosis [48]. In addition valvular abnormalities can be caused by both congenital and acquired diseases such as in bicuspid aortic valves and rheumatic disease.

The mechanisms of age related calcification of the aortic valve has been attributed to progressive degeneration involving dystrophic calcification, which results from injury due to long-standing and repetitive mechanical stress. Although still not fully understood, calcific deposits in the aortic valve are dependent on the biochemistry and structure of the tissue as well as the chemical environment. The relationship between calcification and the process of arteriosclerosis is not clear [49]. A close relationship has been suggested by studies showing calcific deposits associated with lipid accumulations in the aging aortic valves especially in areas of the fibrosa where fibroblasts are diminished in number and where calcification and lipid are associated with fragments of degenerated fibroblasts [50]. It has also been suggested that in aging cells, extra-cellular calcium reacts with membrane phospholipids of deteriorating cells with formation of calcium phosphate in a similar manner as described for bioprosthetic valves in the introduction of this manuscript. A growing number of studies have recently challenged the view that aortic calcification is merely due to aging and have suggested a role for non collagenous extra-cellular matrix proteins such as osteopontin which bind to hydroxyapatite and may play a role in calcification [51-54]. Moreover correlations between calcification, macrophage accumula-
tion and osteopontin expression in aortic valve stenosis have been reported, although the exact role of osteopontin is unclear [55-58].

Patients with congenital bicuspid valves are predisposed to early aortic stenosis due to abnormal leaflet structure leading to fibrosis and calcification [59]. Under 70 years of age, congenital calcified bicuspid valves account for 50% of all cases where as in above 70 years of age, degenerative calcific stenosis account for 48% of cases [60]. Rheumatic disease, which results in fusion of the leaflets, is also a cause of aortic stenosis. Rheumatic aortic stenosis is rarely isolated and usually occurs in conjunction with mitral valve stenosis [59]. Other conditions less frequently associated with aortic stenosis are Paget’s disease, renal disease and ochronosis [61–63].

Aortic stenosis results in chronic left ventricular pressure overload with subsequent ventricular adaptation. Initially the left ventricle compensates for the increased pressure by pumping more forcefully to maintain its cardiac output. With persisting stenosis, the ventricle compensates by increasing its muscular mass whereby it generates compensatory force to maintain cardiac output [64]. Mature cardiac myocytes increase their volume with thickening of the ventricular wall referred to as concentric compensatory hypertrophy [64]. Mature cardiac muscle cells or myocytes do not divide and compensatory hypertrophy occurs by an increase in myocyte volume, which is limited. In untreated, longstanding aortic stenosis compensatory hypertrophy fails leading to dilate cardiomyopathy and cardiac decompensation with a loss of myocytes. When symptoms develop, the prognosis becomes poor and the average survival is 2 to 3 years for patients with symptoms of angina or syncope, and 1 to 2 years for those who develop congestive heart failure [65].

Aortic valve insufficiency may be caused by abnormalities of the leaflets, the aorta, or a combination of both and is characterized by inadequate closure of the valve. Isolated aortic regurgitation is rare and it occurs most commonly in combination with aortic stenosis. When the pathological abnormalities are confined to the aorta, the leaflets remain anatomically normal. The latter patients have progressive dilatation of the aortic sinuses and on occasion dilatation of the annulus of the valve with aortic insufficiency. Most are idiopathic [66] (annulaoartict ectasia) but may be associated with a with a spectrum of pathology which include Marfan syndrome [67], aortic dissection and aortitis [68-69] as well as rare connective tissue disorders such as Reiter’s disease and Ehlers-Danlos syndrome and rheumatoid arthritis [70-73].

Unlike aortic stenosis, both volume and pressure overload of the left ventricle occurs. The compensatory changes of the ventricle involve eccentric hypertrophy. Myocytes do not thicken but add myofibrils in series with an increase in ventricular volume[74]. The exact molecular mechanisms involved remain unknown but recent evidence suggests that stress activates calcium-dependent kinases, which induces
vasoactive peptides (angiotensin II, neuregulin and other growth factors) leading to increased myofibril production and decreased degradation at a cellular level and consequent hypertrophy [75]. Importantly, myocyte hypertrophy in aortic stenosis leads to an increased synthesis of myofibrillar protein, where as in aortic insufficiency the increased myofibrillar protein content is mainly due to decreased degradation [76]. As systolic stress increases and persists in time, the ventricular wall also thickens and develops fibrosis possibly due to ischemia, myofibrillar degeneration or myocyte apoptosis mediated by death proteins such as the mitochondrial death protein Nix [77]. Subsequently, the ventricle becomes over distended at which point the myocardium loses its reserve (wall tension) and the heart decompenses. Although chronic aortic insufficiency is generally well tolerated for years, acute aortic insufficiency, as for instance in the case of acute aortic dissection, is not and can lead to death. The prognosis in chronic patients who become symptomatic is poor with death occurring within 4 years after development of angina and within 2 years after the onset of congestive heart failure [68].

2.3 **Treatment of Heart Valve Disease.**

Treatment for valvular heart disease depends on the type and severity of the disease and involves either medical or surgical therapy. Medical therapy in patients with aortic insufficiency is not able to significantly reduce regurgitant volume due to the valvular incompetence and because of the low diastolic pressures that are observed due to the failure of the valve to close sufficiently. As such medical therapy is usually directed at reducing the systolic pressure (hypertension) in an attempt to reduce the stress in the ventricular wall (wall stress) and ease distension and as such to improve ventricular function. The drugs of choice are vasodilators, which have been shown to be beneficial in delaying surgery, or treating patients who are not candidates for surgery [79-81]. In patients with symptomatic aortic stenosis, medical treatment is usually reserved for patients who are not candidates for operation since the treatment of choice is surgical. In these patients pharmacological therapy is used only as adjunctive treatment for complications associated with aortic stenosis such as cardiac failure (e.g. angiotensin converting enzyme inhibitors, diuretics), fluid retention (diuretics), arrhythmias and hypertension [82-83].

Surgical treatment of aortic valve disease includes reconstructive procedures that do not replace the valve and valve replacement. In neonates and young children with congenital aortic stenosis for instance, surgical techniques are used to mobilize the leaflets and thus allow the child to grow to adolescence at which time other procedures can be employed. In patients with aortic aneurysms or dissection and in whom
the aortic leaflets are not affected, the valve is preserved in some procedures that completely resects the diseased aorta and replace it by synthetic material [84-85] or in other techniques a less radical resection [86] of the aorta is employed and the valve is mobilized and inserted inside a synthetic Dacron tube which is sewn in place to replace the aorta. Both techniques preserve the native valve, the first preserving its distensibility and the second is aimed at preventing future dilatation. Although some diseased aortic valves can be repaired a large number of valves are not suitable for repair and need replacement [87]. Consequently, the main treatment for diseased aortic valves is surgical replacement, which can significantly improve the life expectancy of patients. A 60 year old man for example with severe aortic stenosis has approximately 4 years to live, but after valve replacement his life expectancy increases to approximately 13 years [88]. However, a 60 year old man in the general population has a life expectancy of 18 years which is 5 years longer than that of the valve recipient and it has been suggested that most if not all of this difference is related to the use of current bioprostheses which do not match the native valve and are sub optimal.

**Limitations of Current Prosthetic Valves.**

Current commercial prosthetic valves can be grouped into three categories (Fig 9): (a) bioprostheses, (b) homograft valves and (c) mechanical prosthetic valves. In addition to specific disadvantages, all currently available prosthetic valves are a-vital and lack the ability to grow.

**Bioprosthetic valves**

Bioprostheses are derived from animal or human tissue after chemical treatment. Most are manufactured from porcine valves or bovine pericardium after treatment with the monoaldehyde glutaraldehyde [88] which are available in various configurations and are either affixed to a supporting metal or plastic stent (stented valves) or not (stentless valves). These valves require no anticoagulation therapy [89] and despite disadvantages they have in the past, and continue to serve patients well with a durability of 15 to 20 years [90-94]. While high functional stresses associated with tissue fixation onto the stents can result in accelerated tissue fatigue with resulting tears [95], in presently used bioprosthetic valves, calcific deterioration is the main cause of valve failure [96]. Indeed 20-30% of porcine valves require reoperation within 10 years and 50 % within 12-15 years. Valve failure is more rapid in younger patients (almost 100% within 5 years in patients <35 years of age) [97-99].

Glutaraldehyde treatment of porcine aortic valves for human implantation was first introduced in 1969 [100] and the majority of bioprosthetic valves implanted cur-
Currently are covalently cross-linked with glutaraldehyde which stabilizes the tissue while reducing its antigenicity and sterilizing it to some extent. The limited durability of glutaraldehyde fixed bioprosthetic valves has been attributed to altered mechanical properties, antigenic properties of the cells, glutaraldehyde interactions and the calcification potential of cell membrane [100-104]. In addition, in children, in whom bioprosthetic valve failure is observed more commonly than in adults a possible involvement of high serum phosphate and osteocalcin as well as enhanced metabolism of parathyroid hormone and vitamin D has been suggested [105-107].

While it is widely acknowledged that glutaraldehyde treatment results in a reduction of immunological recognition of the xenogenic tissue and its stabilization to degradative enzymes in humans [108], glutaraldehyde has been suggested to be a cause of pathological calcification [101,109]. In bioprosthetic valves, glutaraldehyde introduces thermally and chemically stable cross-links or Schiff-bases with amino groups in collagen, which become compromised with time and result in a leaching out of glutaraldehyde [110]. Such free aldehydes have been shown to be easily oxidized to carboxylic acid, which is a potential site for calcium binding [111,112]. Glutaralde-
hyde also stiffens and alters the biomechanical characteristics of biological valvular tissue whereby the collagen is locked in geometric configuration. Such alterations are associated with demonstrable changes in leaflet motion which produce abnormal stress patterns causing buckling, accelerated calcification and eventual tissue failure [113]. Moreover it is known that bioprostheses show progressive depletion of proteoglycans both in vitro and in vivo [114,115]. As such because proteoglycans contribute significantly to the viscoelastic properties and accommodation of stresses in the leaflet, their loss may influence mechanical deterioration.

In addition, glutaraldehyde cross-linked valves are and remain non-viable tissues without opportunity for either growth or tissue renewal. Their cells are no longer biologically dynamic and as such are incapable of replenishing and maintaining the extra cellular matrix. After implantation these valves are not repopulated with host cells which at least partially can be explained by the cytotoxicity of glutaraldehyde and the inability of cells to penetrate the cross-linked matrix [110,116]. Furthermore, although stable cross-links in cellular and extra cellular matrix proteins reduce immunogenicity, porcine tissue retains a residual ability to trigger an immune response that can activate macrophages, which in turn can obtain an osteoblast calcium depositing phenotype [117]. Moreover, it is well established that cell and the cellular membrane provoke early calcification of biological heart valves, whereby a direct relation between specific antibody response and the calcification of porcine tissue has been demonstrated [104,118]. Furthermore phospholipids present in cell membranes are believed to separately initiate calcification by forming calcium phosphate crystals with free serum calcium [103,119,120].

To reduce their propensity to calcify manufacturers employ several anti-calcification treatments in the production of bioprosthetic valves. However, they have not yet resulted in significantly improved valve durability (amino-oleic-acid, ethanol) [121].

**Homograft valves**

Human homografts (also referred to as allografts) are intact human valves and the first reported use of a cadaveric homograft to treat aortic valve disease was by Murray in 1956 [103] that implanted valves in the descending aorta of patients. Sir Donald Ross in the Lancet reported the first use of the homograft valves in the aortic or orthotopic position in 1962 [122].

Valves are usually obtained from beating heart donors whose heart are unsuitable for transplantation or from cadavers, and are either stored by cryopreservation
before use [123,124] or are used after antibiotic sterilization within 60 days [125]. At present these valves are most commonly stored by cryopreservation before use as this provides a long shelf life in view of a shortage of donors [126]. In fact a major limitation to the use of homografts is their limited availability especially in the pediatric sizes.

Cryopreserved human valves (homografts) show durability comparable to the best xenografts and can last for 20 years despite no HLA or ABO matching [127]. Although they perform well, these valves are also avital [128,129] and succumb to fibrosis and calcific deterioration.

The occasional survival of viable donor fibroblasts in cryopreserved homografts have been reported and it is likely that some cells persist for at least some time [126]. The functionality of these cells is unknown. Several attempts have been reported to modify homografts by maintaining cellularity [126] and to improve long term structural integrity [130]. However if such valves remained vital, their cell may prove detrimental by inciting their own rejection [131]. Multiple studies have demonstrated the generation of donor-specific alloantibodies directed against human leukocyte antigens (HLA) class I (A and B antigens) and II (DR antigens) in valve recipients [132,133]. However, despite consistent evidence of antibody formation directed against the HLA antigens, the clinical significance of these observations remains unclear [134,135].

Homografts have several advantages and these include excellent hemodynamic profiles [136,137] more similar to native valves, no requirement for anticoagulation and a low rate of infection compared to other prosthetic valves [138,139,140]. In fact in endocarditis they remain the prostheses of choice, especially when the aortic annulus is disrupted by infection. They remain however, a-vital structures with limited durability even after implantation and show structural failure with time, which approximates 19% to 38% at 10 years and 69% to 82% at 20 years. [138,141].

**Mechanical valve prosthesis**

A mechanical valve is constructed from man-made materials (non biological). The first mechanical valve, developed in 1960, was of the “ball and cage” design of Starr-Edwards type. This has proven to be a very durable valve over time [142] and in fact some valves are still functioning today more than 30 years after implantation. However poor biocompatibility owing to damage caused to blood elements and the need for high doses of anticoagulation plagued these valves and led to the search for better designs which led to development of mono leaflet tilting disc valves shown in figure 9. Although these valves show improved hemodynamics, they still required life
long anticoagulation and were sometimes considered noisy by patients [143]. Ever since, mechanical valve design has continuously improved to the newer generation of valves, namely the bileaflet valves. Presently mechanical valves are constructed from materials such as polymers, metal and pyrolytic carbon and they show life long durability and excellent hemodynamics and more than 60% of heart valve replacements are made using mechanical prostheses [144].

Nevertheless, although more durable than bioprosthetic valves, mechanical valves remain less biocompatible. Their surfaces are thrombogenic and their major limitation is the need for anticoagulation with its associated risks in recipients [145,146,147]. They are associated with substantial risk of systemic thromboemboli and thrombotic occlusion largely owing to the non-physiologic surfaces and abnormal flow characteristics produced by rigid occluders [146,148]. Moreover these valves cannot be placed in patients in whom anticoagulation is contraindicated. In young expectant mothers for instance, they pose an increased risk for bleeding complications [149]. In patients who have received a mechanical valve and are on anticoagulation therapy the risk of hemorrhage during long term anticoagulation therapy is 2 to 5 per patient/ year, with cerebral, gastrointestinal or retroperitoneal bleeding being the most common in mechanical valve recipients on anticoagulation [150,151].

A Tissue Engineered Solution.

A tissue engineered aortic valve constructed from scaffolds and cells, will overcome the limitations of currently available prosthetic valves by regenerating and maintaining its extra cellular matrix, require no anticoagulation therapy, avoid immunological rejection and theoretically be capable of growth. The critical issues facing such a valve include where to start in terms of scaffold material and how to accomplish the requisite recellularization. The present study addresses some of these issues, which are at the foundation of developing a tissue engineered aortic valve.

In Chapter 1, we describe calcification in glutaraldehyde aortic valves obtained from kangaroos and pigs and assess the hydrodynamic properties of their respective matrices. In Article I.1, we examined the calcification potential of kangaroo aortic valves for two reasons. Firstly we wanted to check the rate of calcification of these valves compared to that of porcine valves in the rat model and hence their reported potential as bioprosthetic valves. For instance observation of significantly higher calcification compared to porcine valves might rule them out as potential matrices despite other advantages as described later. Secondly the calcification rates in these biological valves could be useful in eventual comparative assessment of the calcification potential of decellularized matrices.
In Chapter 2 we illustrate the nature of calcific deposits within the leaflet layers of an explanted bioprosthetic valves in an attempt to understand their destructive effect on the extra cellular matrix. This study is part of an explant analysis study initiated in our department to better understand the mode of failure of bioprosthetic valves and aid us in avoiding similar pitfalls in engineering a valve. This study was unique in that it afforded us the rare opportunity to evaluate different modes of failure in the same valve, namely calcific and non calcific destruction of the matrix and report it for the first time. It was obvious that calcification was strongly associated with the extra cellular matrix. Such information could also prove useful in assessing eventual calcific destruction in tissue engineered valves or matrices.

Satisfied that kangaroo aortic valves had a comparable rate of calcification to porcine valves, we proceeded to develop matrices of both types. In Chapter 3 the hydrodynamic properties of porcine and kangaroo aortic matrices prepared from aortic valves are evaluated. While porcine xenogenic matrices are currently used in tissue valve engineering, ours is the only group reportedly investigating kangaroo aortic valve matrices and to justify their use we needed to ensure satisfactory hydrodynamic performance, at least compared to similar porcine matrices, since this was not done before.

In Chapter 4, we compared the biomechanical properties of porcine aortic valves before and after cryopreservation and that of non-decellularized leaflets. The decellularization procedure employed and described was time consuming (5 days). It became obvious to us that using our protocol, it would not be practical nor efficient to prepare matrices each time one is need and especially so in a clinical setting. We therefore investigated the feasibility of preserving such matrices by cryopreservation. We evaluated the mechanical effects of both decellularization and cryopreservation on matrices.

In Part III, we describe a model for transdifferentiating fibroblasts to myofibroblasts for use in tissue valve engineering and the use of growth factor to modulate their proliferative and invasive properties (Chapter 5 and Chapter 6). Such manipulations allow us to have sufficient quantities of myofibroblasts in order to seed matrices and to enhance their repopulation.

In Chapter 7, we investigated the inherent calcification potential of both porcine and kangaroo matrices in sheep. Information from such studies could be useful in improving the quality of matrices and in understanding eventual failure of tissue engineered valves.
References


Part I  
Matrix Procurement

To start with, a matrix, which replicates functionally if not structurally, the performance of the native aortic valve needs to be selected. Two main types of scaffold material are currently being investigated, namely decellularized biological valve matrices and those fabricated from biodegradable polymers [1, 2]. A polymer based approach must replicate the performance characteristics of the native aortic valve and no such polymer has yet been developed [3]. Secondly, although polymers have been applied in the tissue engineering of bone and skin, present knowledge into their use in tissue valve engineering is insufficient to prevent a foreign body fibroblast reaction rather than establish a normal tri-laminar structure with appropriate proportions of extracellular matrix, proteins and multiple cell types with density and location characteristic of a functional aortic valve [4]. Biological matrices derived from porcine valves on the other hand, avoid many engineering design problems and are used on the assumption that a decellularized xenograft scaffold, devoid of cells is potentially less immunogenic than cellularized bioprosthetic valves [5], which have to be treated with glutaraldehyde before implantation.

While biodegradable polymer and biological scaffolds have been tested in vivo, alternative material being tested for use as scaffolds in tissue valve engineering includes, collagen, hyaluronan and fibrin. A major problem with the use of reconstructed collagen as scaffolds is the observation that within such scaffolds, cells rapidly enter apoptosis. Giachelli and colleagues have reported that combining collagen with chitosan, which is derived from crustacean shells, show an improvement compared to the use of collagen alone [6]. Others, [7] reported satisfactory proliferation of myofibroblasts in collagen scaffolds when these cells are cultured in combination with endothelial cells. More recently by varying the concentration of collagen, Taylor and co-workers, using the technique of Rapid Prototyping, which can potentially allow for the development of three dimensional scaffolds, have shown that scaffolds containing 1% of collagen allowed cell proliferation to a greater extent than those based on 2% or 5% collagen [8].

The visco-elastic properties and biocompatibility of the glycosaminoglycan hyaluronan [9, 10] has led to investigations of its use as a potential scaffold in tissue valve engineering. Vesely and co-workers, working with hyaluronan gels, cross-linked with divinyl sulfone [11], have reported that such constructs have elastic properties comparable to the aortic valve cusps and might find use in the central fibrosa portion of
cusps, possibly combined with collagen in one construct. As an alternative to collagen, a number of investigators are also considering the use of fibrin gels [12].

Collaborative work between the Departments of Cardiac Surgery, University Hospital Ghent and the Departments of Cardiothoracic Surgery of Fremantle Hospital, University of Western Australia, Perth and The Prince Charles Hospital, University of Queensland, Brisbane, Australia, has led to the use of kangaroo heart valves as an alternative source of biological matrices.

Kangaroos are upright and more similar in posture to humans compared to pigs. And their aortic valves are morphologically and histologically different from porcine aortic valves. In particular, kangaroo aortic valves have thinner leaflets with a more compact collagenous matrix compared to porcine aortic valves and unlike porcine aortic valves they have no muscular shelf in the right coronary cusp [13, 14]. Such a muscular shelf, in addition to partially obstructing flow, also necessitates muscular decellularization when preparing a-cellular biological scaffolds. Its absence in the kangaroo valve avoids both possible obstruction with hydrodynamic consequences as well as the need for muscle decellularization. Indeed, Weinhold and co-workers who implanted whole kangaroo aortic valves in the tricuspid position of sheep [14] reported superior hemodynamic performance of kangaroo aortic valves compared to porcine aortic valves. A thinner kangaroo leaflet matrix might also require less cells to repopulate. Investigations in our laboratory confirmed that while similar structural elements are present in kangaroo and porcine valves, their distributions in the fibrosa, ventricularis and spongiosa of the leaflets are different in these two species, with kangaroos having a thinner spongiosa layer and a relatively thicker ventricularis layer.

While there is no standardized method, typically, matrices are prepared from biological valves by cell lysis using alternating treatments of hypertonic and hypotonic solutions followed by detergent extraction and enzymatic digestion [15].

References


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Chapter 1

Kangaroo vs. Porcine Aortic Valves: Calcification Potential after Glutaraldehyde Fixation

K. Narine, Cyrille C. Chéry, Els Goetghebeur, R. Forsyth, E. Claeys, Maria Cornelissen, L. Moens, G. Van Nooten
Kangaroo vs. Porcine Aortic Valves: Calcification Potential after Glutaraldehyde Fixation

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Key Words
Kangaroo · Porcine · Aortic valves · Calcification

Abstract
The aim of this study was to evaluate and compare the calcification potential of kangaroo and porcine aortic valves after glutaraldehyde fixation at both low (0.6%) and high (2.0%) concentrations of glutaraldehyde in the rat subcutaneous model. To our knowledge this is the first report comparing the time-related, progressive calcification of these two species in the rat subcutaneous model. Twenty-two Sprague-Dawley rats were each implanted with two aortic valve leaflets (porcine and kangaroo) after fixation in 0.6% glutaraldehyde and two aortic valve leaflets (porcine and kangaroo) after fixation in 2% glutaraldehyde respectively. Animals were sacrificed after 24 h and thereafter weekly for up to 10 weeks after implantation. Calcium content was determined using inductively coupled plasma-mass spectrometry and confirmed histologically. Mean calcium content per milligram of tissue (dry weight) treated with 0.6 and 2% glutaraldehyde was 118.2 and 110.4 μg/mg tissue for kangaroo and 95.0 and 108.8 μg/mg tissue for porcine valves. Calcium content increased significantly over time (8.8 μg/mg tissue per week) and was not significantly different between groups. Regression analysis of calcification over time showed no significant difference in calcification of valves treated with 0.6 or 2% glutaraldehyde within and between the two species. Using the subcutaneous model, we did not detect a difference in calcification potential between kangaroo and porcine aortic valves treated with either high or low concentrations of glutaraldehyde.

Introduction
The majority of commercially available biological valve prostheses are made from porcine aortic valves or bovine pericardium after fixation in low concentrations of glutaraldehyde (1,5-pentane dialdehyde; CHO(CH$_2$)$_3$CHO) [1, 2]. Although the precise mechanism is still controversial, calcific deterioration is a major cause of contemporary bioprosthetic heart valve failure [3, 4].

Carpentier et al. [5] reported significant differences amongst different donor and recipient species with regard to calcification of biological tissue. Weinholdt et al. [6] and more recently Neethling et al. [7] reported on the potential of kangaroo aortic valves to calcify less than porcine aortic valves in vivo, using the sheep model.

Subcutaneous implantation of bioprosthetic valves in the rat model is still commonly used to investigate calcification potential. Apart from its low cost and simplicity, this subcutaneous model is attractive because the bio-
logical and morphological features of intrinsic leaflet calcification seem have been reported to be analogous to those seen in clinical explants but at an accelerated rate [8–10].

The use of glutaraldehyde as the preserving agent of bioprosthetic heart valves became widespread three decades ago after it was reported that fixation of porcine heart valves in this agent resulted in stable cross-links and rendered the tissue essentially non-immunogenic [11, 12]. Several reports, however, have implicated glutaraldehyde in the calcification process of bioprosthetic heart valves [13, 14]. In order to avoid excessive glutaraldehyde, current bioprosthetic valves are fixed in low concentrations (<1%) of glutaraldehyde. Despite the foregoing and the use of antimicrobial treatments in tissue valve manufacture, the long-term durability of glutaraldehyde-treated bioprosthetic valves continue to be limited by their propensity to calcify [15–17].

The reported low calcification potential of glutaraldehyde-fixed kangaroo valves in the sheep circulatory model prompted us to compare the calcification potential of kangaroo and porcine aortic valves after glutaraldehyde fixation in the rat subcutaneous model. Moreover, to evaluate a possible influence of the concentration of glutaraldehyde used, we evaluated valves after fixation in low and high concentrations of glutaraldehyde, namely 0.6 and 2.0% phosphate-buffered glutaraldehyde solutions.

Materials and Methods

Animals

Twenty-two Sprague-Dawley rats weighing approximately 150 g each and 6 weeks old were obtained from Harlan Laboratories (Horst, The Netherlands). Animals were allowed to acclimatize to the Animal Facilities at our institution for 1 week before the investigations. All animal care complied with The Guide for the Care and Use of Laboratory Animals, 1996 [18]. All of our protocols involving animals were approved by the Ethical Commission for Animal Experiments of the University and the University Hospital of Ghent (Project No. ECP 022).

Valve Leaflet Procurement and Fixation

Porcine valves (n = 15) used in this study were obtained from the slaughterhouse of the Department of Animal Production, University of Ghent. Immediately after slaughter, hearts were retrieved and placed on wet ice for transportation to the Laboratory of Experimental Cardiac Surgery, University Hospital, Ghent. Aortie valves (n = 15) from Western Grey kangaroos (Macropus fuliginosus) were harvested and supplied under law by a member of the Professional Shooters Association of Western Australia. After harvest, hearts were placed on ice and transported to the Fremantle Heart Institute, University of Western Australia, from where they were shipped by air to our laboratory after glutaraldehyde fixation. Before fixation, each heart was rinsed with cold saline (0.9% NaCl) before valves were dissected free and again rinsed in cold saline.

Methods

A total of 44 kangaroo and 44 porcine leaflets were implanted. Valves in each group were randomized. Twenty-two leaflets of each type were fixed in phosphate (K$_2$HPO$_4$)-buffered (pH 7.4) 0.6% glutaraldehyde. The remaining 22 leaflets of each type were fixed in 2% glutaraldehyde in the same buffer. Fixing solutions were prepared from a stock solution of 25% glutaraldehyde (Merck, Darmstadt, Germany). Potassium phosphate (K$_2$HPO$_4$) was also purchased from Merck. The final pH (7.4) of both concentrations of glutaraldehyde was achieved by titration using 0.5 M NaOH. Leaflets were fixed for 7 days, after which they were transferred to a solution of 0.2% glutaraldehyde in the same buffer and stored at 4°C until further use.

Implantation and Explantation of Leaflets

All animals were anesthetized using a combination of ketamine (0.9 mg/kg), xylazine (0.0075 mg/kg) and atropine (0.0001 mg/kg). Following anesthesia, four subcutaneous pouches were created in each animal, one in each abdominal quadrant. Leaflets were implanted in each animal as follows. In the right upper quadrant, one kangaroo leaflet fixed in 0.6% glutaraldehyde and in the right lower quadrant, one kangaroo leaflet fixed in 2% glutaraldehyde. In the corresponding upper and lower quadrants on the left side, porcine leaflets fixed in 0.6 and 2% glutaraldehyde were implanted respectively. All leaflets were thoroughly rinsed to remove excess glutaraldehyde before implantation. After recovery from anesthesia, all animals were returned to the animal facilities and fed a standard rat diet. Two animals were sacrificed after 24 h, and then another 2 weeks for up to 10 weeks. After sacrifice, each leaflet was divided into segments by cutting from the free edge down towards the base. One segment was taken at random, frozen at −80°C and kept for quantitative calcium determination. Another segment was fixed in 4% formaldehyde for histological examination.

Histology

Specimens of non-implanted (fresh) and implanted kangaroo and porcine aortic valve leaflets were examined histologically. Fresh specimens were fixed in 4% phosphate-buffered formaldehyde and stained with Masson’s trichrome to illustrate the gross histological structure. Explanted samples for light microscopy were fixed immediately in 4% phosphate-buffered formaldehyde. Calcific deposits were identified with von Kossa stain.

Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)

In this work, a quadrupole-based ICP-MS [19–21] (Elan DRC plus, PerkinElmer-SCIEX, Concord, Canada) was used. This equipment contains a dynamic reaction cell, in which selective ion-molecule chemistry allows the reduction of interferences that the analyst might suffer from. As such, the technique allows for the detection of significantly lower concentrations of calcium (approx. 1,000–6,000) than classic atomic absorption spectrometry. In our application, this technology permits the elimination of the argon ions ($^{40}$Ar$^+$), which may interfere with the determination of calcium (Ca) via the signal of $^{40}$Ca.$^+$

Explanted samples were thawed in a clean room, Class 100. Every precaution was taken to avoid sample contamination. Samples were washed twice with MilliQ water (doubly distilled water which was further purified using a MilliQ water purification system (Millipore, Bedford, Mass., USA)). Tissues were lyophilized and the dry weight determined before undergoing microwave oven-assisted acidic digestion (Microwave Digestion System: MLS-1200 MEGA Technology, Milestone, USA, with MDR microwave digestion rotors), used with tetrafluormethanol vessels). Samples plus 1 ml 14 M HNO₃ (purified by sub-boiling in quartz equipment), 0.2 ml H₂O₂, 3 ml H₂O, 100 µl of 50 mg/l cobalt (Alfa, Karlsruhe, Germany). The samples in solution were diluted to 100 ml using MilliQ water.

Measurements were performed on the sample solutions after being diluted another 100 times. Cobalt is used as the internal reference for all ICP-MS measurements. Blank solutions and external standards were prepared in an analogous manner to the samples. Calcium concentrations were expressed in micrograms of calcium per milligram of tissue (dry weight).

**Statistical Analysis**

Calcium content over time was analyzed using regression methods and analysis of variance (ANOVA). Two groups of 11 animals yielded duplicate observations on calcium content in porcine and kangaroo leaflets treated with 0.6 and 2% glutaraldehyde as 1 animal per group was sacrificed at 24 h after implantation and then weekly for 10 weeks. We fitted regression models which allowed the mean response to vary with time, species and glutaraldehyde concentration. We also looked at possible interaction effects between these prognostic factors. Time, and only the time variable, contributed significantly to the model. Once this was verified, we adjusted all treatment comparisons for the time effect. This allowed us to analyze average outcomes as well as variations over the weeks. Hence, when we report mean differences, these differences are present at fixed values of time. Calcium content was regressed on time for kangaroo and porcine valves fixed in 0.6 and 2% glutaraldehyde, respectively. To compare calcium concentrations between kangaroo and porcine valves, we considered per animal the difference in calcification of its implanted kangaroo and porcine valves at each concentration of glutaraldehyde. These paired differences were then regressed on time, on the concentration of glutaraldehyde and on their interaction. The final model was decided upon by performing a backward stepwise regression procedure (with p = 0.05 for retention).

A similar analysis was performed to compare results at different concentration levels of glutaraldehyde in the same species of valve. Missing data were treated as missing completely at random.

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**Fig. 1.** Representative histological preparations of the body of the leaflet of fresh kangaroo (K) and porcine (P) aortic valve leaflets (Masson’s trichrome stain). F = Fibrosa; s = spongiosa; v = ventricularis. Collagen stains blue. x 100.

**Fig. 2.** Representative histological preparations of kangaroo (K) and porcine (P) after explantation (von Kossa stain) at 24 h (K1 and P1), 7 weeks (K2 and P2) and 14 weeks (K3 and P3) showing the body or central portion of the leaflets. Calcium stains black. x 100.
Results

Histology

Figure 1 shows histological preparations of unimplanted kangaroo and porcine leaflets. In both species a fibrous layer or fibrosa is located on the aortic surface of the leaflet and the less fibrous ventricularis layer is located on the ventricular or inflow surface. Separating the fibrosa and ventricularis is a spongiosum layer or spongiosa which is thicker in the porcine leaflets. The density of collagen bundles in the fibrosa is more pronounced in the kangaroo valve leaflets when compared with porcine valve leaflets. In addition, the ventricularis layer in kangaroo valves is thicker than in porcine valves and more staining of collagen was observed in this layer in kangaroo leaflets. Von Kossa stain of explants confirmed calcification in both porcine and kangaroo leaflets. Morphologically, we observed no difference in calcification in comparable explants over time. In both types of explants we observed calcifications in all three layers of the leaflet but more extensive in the spongiosa and ventricularis. Figure 2 shows representative von Kossa preparations for porcine and kangaroo leaflets explanted at 24 h, 7 weeks and 10 weeks.

Calcification

The raw data for calcium content in explanted leaflets are shown in table 1a and are statistically summarized in table 1b. Figure 3 shows the regression lines per type of valve for this raw calcium data; calcium content is expressed in micrograms of calcium per milligram of tissue (dry weight) (µg Ca/mg tissue).

Table 1. Raw data of the average calcium content in explanted leaflets (a) and statistical summary of the raw data shown (b)

a

<table>
<thead>
<tr>
<th>Explan</th>
<th>Average calcium content, µg Ca/mg dry weight of tissue</th>
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<tbody>
<tr>
<td></td>
<td>P0.6</td>
</tr>
<tr>
<td>24 h</td>
<td>1.75</td>
</tr>
<tr>
<td>1 week</td>
<td>46.1</td>
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<td>2 weeks</td>
<td>104.6</td>
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<td>65.6</td>
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<td>4 weeks</td>
<td>106.8</td>
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<td>6 weeks</td>
<td>180.6</td>
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<td>7 weeks</td>
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<tr>
<td>8 weeks</td>
<td>75.0</td>
</tr>
<tr>
<td>9 weeks</td>
<td>120.0</td>
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<tr>
<td>10 weeks</td>
<td>138.5</td>
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b

<table>
<thead>
<tr>
<th></th>
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<tr>
<td>Number</td>
<td>19</td>
<td>22</td>
<td>19</td>
<td>20</td>
</tr>
<tr>
<td>Mean</td>
<td>94.9842</td>
<td>116.1500</td>
<td>106.7947</td>
<td>110.3500</td>
</tr>
<tr>
<td>Standard error of mean</td>
<td>12.48984</td>
<td>15.58478</td>
<td>11.83288</td>
<td>7.53473</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>54.44196</td>
<td>73.09911</td>
<td>51.57835</td>
<td>33.69633</td>
</tr>
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<tr>
<td>75</td>
<td>138.0000</td>
<td>167.7500</td>
<td>144.1000</td>
<td>128.0000</td>
</tr>
</tbody>
</table>

P = Porcine, K = kangaroo, 0.6 and 2.0 represent different glutaraldehyde concentrations.
Table 2. Linear regression model of the raw data (a) and summary statistics for the paired differences in calcium concentration of valves treated with 2% and 0.6% glutaraldehyde (b).

<table>
<thead>
<tr>
<th>Model</th>
<th>Unstandardized coefficients</th>
<th>Significance</th>
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<tr>
<td></td>
<td>B</td>
<td>standard error</td>
<td></td>
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<tr>
<td>1 (Constant)</td>
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<tr>
<td>Time</td>
<td>8.790</td>
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<tr>
<td>Porcine</td>
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<tr>
<td>Dose</td>
<td>0.972</td>
<td>10.635</td>
<td>0.927</td>
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**b**

<table>
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<th></th>
<th>P2.0-P0.6</th>
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<th>P0.6-K0.6</th>
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<td>20</td>
<td>19</td>
<td>18</td>
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<td>12.3426</td>
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<td>-8.0000</td>
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<tr>
<td>Standard deviation</td>
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<td>75</td>
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<td>50</td>
<td>4.5000</td>
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<td>30.1250</td>
<td>30.2500</td>
<td>29.0000</td>
<td>26.7500</td>
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</table>

K = Kangaroo valves; P = porcine valves; 2.0 and 0.6 represent glutaraldehyde concentrations.

Analysis of porcine and kangaroo valves treated with 2% and 0.6% glutaraldehyde, respectively. The stepwise backward regression (with p = 0.05 for retention) retained no predictors in this model. A similar analysis for differences in calcification between the porcine and kangaroo valves gave similar results: no significant predictors remained in the model and the global average difference shown in table 2b is not significantly different from zero. As the mean difference in calcification between the porcine and kangaroo valves (adjusted for time and level of glutaraldehyde) has an estimated error of 10.6, the non-significant difference is not surprising. Most striking indeed are the large variations seen in outcomes between and within animals.
Discussion

In our subcutaneous model, kangaroo and porcine valves show an increase in calcification with time and confirm a trend which has been seen in porcine aortic valve leaflets [9]. There was, however, no significant difference in the amount of calcification seen in porcine and kangaroo leaflets treated with 0.6 or 2% glutaraldehyde over time. Neethling et al. [7] reported lower calcification in kangaroo aortic valve leaflets implanted subcutaneously in rats and explanted after 8 weeks. However, from our observations, while the calcium content in explanted leaflets may differ at individual time points, there was no significant difference in the progression of calcification over time in the two species. Considering the small sample sizes involved, we must realize when interpreting the p values that the data show a large variation in outcomes and little suggestion of an important possible difference between kangaroo and porcine valves in this setting. From our observations, 2% glutaraldehyde fixation did not result in significantly more calcification than 0.6% glutaraldehyde in leaflet tissue in either porcine or kangaroo aortic valves. While this study did not evaluate glutaraldehyde concentrations >2%, it does indicate that at least at a concentration of 2%, glutaraldehyde did not result in significantly more calcification in the subcutaneous model than a concentration of 0.6%. Indeed, Zilla et al. [22], who investigated the effect of high glutaraldehyde concentrations on calcification of aortic wall tissue, suggested that high glutaraldehyde concentrations of glutaraldehyde reduce rather than increase tissue calcification potential. Although our study investigated aortic leaflet tissue, it is in keeping with the findings of the latter authors in that there was no significant increase in tissue calcification with higher glutaraldehyde concentration in our model.

In the sheep circulatory model, Weinholdt et al. [6] as well as Neethling et al. [7] reported less calcification of the kangaroo aortic valve. Interestingly, our subcutaneous static model did not reflect the findings reported in the circulatory model. The correlation between calcification in the rat subcutaneous and circulatory models has been questioned by several authors [10]. A possible explanation of the different observations in the two models is that subcutaneously implanted tissues are not in a circulatory system and are thus not subjected to the same mechanical forces. This argument was reinforced by Carpentier et al. [11] who demonstrated that iron pretreatment mitigated calcification of aortic valves in the rat subcutaneous model, but appeared to promote calcification when tested in the sheep circulatory model. Several authors have suggested that mechanical forces associated with the circulatory model might significantly contribute to tissue valve calcification [23–25]. Vyawahare et al. [24] suggested that fatigue induced damage to type I collagen and loss of glycosaminoglycans are major contributing factors to material degeneration in bioprosthetic valves. In our group, Van Nooten et al. [25] emphasized the role of mechanical stresses by implanting bioprosthetic valves asymmetrically in the sheep model. These authors concluded that abnormal mechanical stresses created by the implantation technique adversely affected calcification potential. It is thus possible that mechanical stresses, not tested for in the subcutaneous model, can significantly influence calcification of leaflets in the circulatory model. Furthermore, Meuris et al. [26] who studied the effects of recipient species, environmental factors and cellularity on the calcification of aortic wall tissue in the rat subcutaneous and sheep models suggested that blood contact in the sheep circulatory model may also influence calcification.

The mechanical behavior of tissue in a dynamic model has been suggested to influence tissue deterioration. Vesely et al. [27] investigated the bending properties of glutaraldehyde-fixed bovine pericardium and porcine aortic valves subjected to mechanical forces. These authors concluded that bovine pericardium demonstrated less compressive buckling than porcine aortic valves when bent. As a possible explanation of this observation, the latter authors suggested that bovine pericardium requires larger forces to bend after glutaraldehyde fixation due to its dense and tightly layered structure without a spongiosa layer. In contrast, porcine valves are looser and can undergo compressive collapse of the spongiosa layer after fixation. Such a structure requires less force to bend and its layers are more likely to undergo compressive buckling than bovine pericardium. Repetitive buckling could lead to structural deterioration and subsequent calcification. Kangaroo valves have a denser arrangement of their collagen fibers, with a thicker ventricularis, and a thinner spongiosa layer than their porcine counterparts. As such, one possible contributing factor to less calcification in kangaroo valves in the circulatory model compared to porcine aortic valves might be less compressive buckling in the denser kangaroo tissue (fig. 1).

We also observed large variations in leaflet calcification between animals. Variations in the calcium content of aortic valves implanted in the rat subcutaneous model are also evident in the literature. In particular, mean calcification levels of 130.0 and 177.8 μg/g were reported by Schoen...
et al. [9] and Hirsch et al. [28] in similar studies using glutaraldehyde-fixed porcine aortic valve leaflets. Makol and Vesely [10] who examined in vivo and in vitro models of calcification suggested that in the rat subcutaneous model the highest variability of calcification likely resulted from the rat subcutaneous conditions or the initial condition of producing calcification. In addition to rat variability, we have also observed within-animal variations in this model.

4 Conclusion

This study compares for the first time the rate of calcification in kangaroo and porcine aortic valve leaflets in the rat subcutaneous model. We could not establish a significant difference in progressive calcification in kangaroo and porcine aortic valve leaflets in our study after 36 weeks of storage at 37°C in a humid environment.

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Calcification of Kangaroo vs Porcine Aortic Valves

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Chapter 2
Scanning Electron Microscopic Surface Topography of Ablation Catheter Perforations and Calcific Tear in an Explanted Bioprosthetic Heart Valve

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Scanning Electron Microscopic Surface Topography of Ablation Catheter Perforations and Calcific Tear in an Explanted Bioprosthetic Heart Valve

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ABSTRACT The authors report a triptom of leaflet destruction in a bioprosthetic aortic valve explanted at 12 years after iatrogenic ablation catheter perforation in a patient who underwent coronary artery bypass surgery and multiple ablative procedures in the interim. Lesions were examined topographically by scanning electron microscopy. Calcium content was evaluated by mass spectrometry and Von Kossa staining. Leaflets exhibited little calcification, except at the commissures of the valve. Scanning electron microscopy revealed distinct lesion topography. The authors present the scanning electron microscopic characteristics of these lesions and of an incision into the valve made for comparison using a pair of scissors. This is believed to be the first report of scanning electron micrographs of ablation catheter perforations and a calcific tear in the same explanted valve. The findings provide a source for comparison in the etiological determination of explanted bioprosthetic valve lesions using scanning electron microscopy.

KEYWORDS ablation catheter, leaflet perforation, porcine bioprosthesis

The majority of currently available bioprosthetic heart valves are made from glutaraldehyde-treated porcine heart valves or bovine pericardium. Thirty to forty percent of the estimated 250,000 heart valve prosthesis implanted worldwide are biological in origin, with the remainder being mechanical prosthesis fabricated from nonbiological material [1]. The basic histological structure of porcine aortic valve leaflets has been described in detail [2]. Each leaflet is made up mainly of collagen, proteoglycans and elastin. These components form 3 main layers: a collagen-rich fibroa layer on the outflow surface (facing the aortic lumen); a less dense collagenous layer, the ventricularis (facing the ventricular cavity), containing elastin fibers; and a proteoglycan rich layer, the spongiosa, which separates the fibroa from the ventricularis. Covering the outflow and inflow surfaces of each leaflet is a monolayer of endothelial cells.
Porcine valves undergo degenerative structural changes as a result of processing for use as bioprosthetic valves and after implantation [3, 4]. Consequently, the main disadvantage of bioprosthetic heart valves compared to mechanical heart valve prosthesis is their limited durability, with reported failure rates of 1% within the first year, 20–30% within 10 years, and more than 50% within 15 years [5, 6]. Structural deterioration is due mainly to calcification and mechanical tissue fatigue over time [7, 8]. In addition to calcification and mechanical degradation, tears in bioprosthetic valves can result from trauma caused by catheters during intravascular procedures such as ablation and cardiac catheterization and from accidental tissue injury during implantation [9, 10].

We report here the scanning electron microscopic findings of perforations caused by an ablation catheter and of a calcific tear in the same porcine bioprosthetic valve and thereby allowing comparison of the collagen architecture in the same prostheses. To our knowledge there has been no report of scanning electron microscopy of an ablation catheter perforation and calcific leaflet tear in the same explanted bioprosthetic valve. Furthermore, for comparative purposes with leaflet injury during implantation we present the scanning electron micrograph of the surface of an incision made into a leaflet of the same valve after explantation using a pair of scissors.

FIGURE 1 A bioprosthesis similar to that described in this study before implantation (St. Jude Medical Toronto SPV valve): (a) shows a lateral view and (b) the inflow surface.

CASE HISTORY

Our patient is a 70-year-old male who underwent aortic valve replacement (St. Jude Medical (TM), 29-mm, Toronto Bioprosthesis) 12 years earlier for aortic stenosis. This heart valve prosthesis is a stentless bioprosthesis comprising a glutaraldehyde-fixed porcine aortic valve covered on the outer surface with Dacron cloth (Figure 1). His cardiac comorbidity included quadruple coronary artery bypass grafting 2 years previously, persistent atrial fibrillation with frequent ventricular extrasystolic beats, for which he underwent multiple ablations procedures, and implantation of a cardiac pacemaker in the interim period. Before his last ablative procedure he was diagnosed to suffer from moderate aortic regurgitation. Cardiac risk factors included a history of smoking, arterial hypertension, and a stressful lifestyle. His only noncardiac comorbidity was inflammatory bowel disease (ulcerative colitis). During his last ablation procedure the ablation catheter was introduced intravenously and the bundle of His was ablated by passing the catheter transeptally from the right to the left ventricular cavity and approaching the bundle of His, and thereby also the aortic valve, by advancing the catheter upward from the ventricular cavity. This procedure was complicated by iatrogenic ablation catheter perforation of the aortic valve resulting in massive aortic regurgitation for which the patient was referred for urgent aortic valve replacement.

At presentation he was in clinical heart failure (NYHA IV). Cardiac echo-Doppler evaluation showed severe aortic regurgitation and a dilated left ventricle (end diastolic volume 157 mL and end systolic volume 86 mL). There was no history of recent infection or substance abuse except for nicotine previously. His chest x-ray was consistent with congestive heart failure, and blood examinations were unremarkable with normal cardiac enzymes. Catheterization and coronary angiography revealed patent coronary artery grafts except for one saphenous vein graft to a small circumflex artery and confirmed a dilated left ventricle with an end diastolic pressure of 20 mm Hg.

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The regurgitant bioprosthetic valve was surgically replaced by a mechanical prosthesis valve (ATS 27 mm, ATS Medicin, Minneapolis, MN, USA) via a median sternotomy under moderate hypothermia and total cardiopulmonary bypass. The patient’s postoperative recovery was uneventful and the hospital stay was 7 days. At 1 month follow-up, he was doing well with a normally functioning mechanical prosthesis.

FIGURE 2. Macroscopic view of the explanted valve. Note the calcific tear (ct) in the right coronary leaflet (RC), the perforation (p1) in the left coronary leaflet (LC), and the perforation (p3) at the base of the non coronary leaflet (NC).


Lesion Topography in a Porcine Bioprosthesis
MATERIALS AND METHODS
Macroscopic Examination
The explanted valve was examined macroscopically and digitally photographed.

Scanning Electron Microscopy
Each lesion was excised. Specimens were fixed in 4% formaldehyde for at least 48h before dehydration using a series of gradually increasing ethanol solutions (30, 50, 75, 95, 100%) during 48h. Specimens were then kept overnight in absolute ethanol. Specimens were dried according to the standard critical-point drying procedure using liquid CO₂ (Blazers CPD 020). Dried specimens were individually mounted on standard stubs and sputter coated with gold (Blazers SCD 040) before examination using a JEOL (JSM 640, JEOL, USA) scanning electron microscope.

Calculus Content
A routine calculus content in the explanted valve was determined. A portion of the body of the right coronary leaflet was excised and examined. Calcification was estimated using a quadruple-based inductively coupled mass spectrometer (ICP-MS) (Elan DRC plus, Perkin Elmer-SCIEX, Concord, Canada) [11]. Tissues were freeze-dried and hydrolyzed with nitric acid. Calcium concentration was expressed in micrograms of calcium per milligram of tissue (dry weight). Another specimen of the same leaflet was fixed in 4% glutaraldehyde, embedded in paraffin, and stained with Von Kossa stain for calcium.

RESULTS
In describing the results the inflow and outflow surfaces of the valve in the normal anatomic position are referred to as the ventriculitis and fibrosa, respectively. The commissures form the highest areas of attachment of the semilunar leaflets to the aortic wall and comprise the attachment of two adjacent leaflets and the associated aortic wall. By convention, the leaflets located next to the ostia of the right and left coronary arteries are referred to as the right and left coronary leaflets, respectively. The third leaflet is referred to as the noncoronary leaflet. Figure 1 shows an unimplanted bioprosthetic valve similar to the explant described in this study.

Calcium Content
The calcium content of the sample taken from the right coronary leaflet was 0.45 μg/mg dry weight of tissue. Von Kossa staining of the body of the leaflet

FIGURE 4 (A, 1920). Surface topography at low magnification of the surface of an incision made into the leaflet using a scissors. Note the uniform calcium architecture and absence of any large calcific deposits.

FIGURE 5 Scanning electron microscopy of the tear located in the right coronary leaflet near to its attachment to the commissure it shares with the left coronary leaflet. (A): Low-magnification view of the inflow surface (Kext B) of the tear. The elevated areas (arrowheads) represent calcific deposits. The numbers indicate the areas that were scanned in more detail. The woven structure above (support) is a support used to position the valve. (B, 1922) Low-magnification overview of one side of the tear. The numbers indicate the areas that were scanned in more detail. (C, 1937) Higher magnification of the inflow surface (A). Note the rougher surface. (D, 1933) Note the frayed bundles of collagen that appear clumped (arrow). (E, 1925) Note the complete absence of the collagen matrix (*) around the large calcific deposit (arrowhead). (F, 1926) Note the smoothness of the calcific deposit (arrowhead), a possible indication of a long-standing lesion. Note also the location of the deposits between the collagen bundles. Note the clumped bundles of collagen with a loss of the parallel collagen structure. (G, 1929) Higher magnification of the surface of the tear. Note the crystals of calcium (arrowhead), the fibrinous deposit (arrow), and the complete destruction of the collagen architecture. (H, 1930) A higher magnification of the area around the tip of the tear. Note the loss of the collagen architecture and the calcific deposits (arrowhead) with what appears to be fibrin deposits.

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(not shown) revealed minimal staining for calcium in the body of the leaflet.

**Macroscopic Examination**

The macroscopic findings are illustrated in Figure 2. The explanted valve showed only slight calcification except at the commissural attachments where calcium deposits were obvious. The right coronary cusp showed a calcific tear (ct) from the commissure between the right and the left coronary leaflets. The left coronary leaflet showed 2 perforations (p1 and p2) in the central portion of the leaflet. A third perforation (p3) was observed at the base of the noncoronary cusp. Figure 3 shows computer-enhanced photographic images of the individual lesions.

**Scanning Electron Microscopy**

Figure 4 shows a low magnification of the surface of an incision made into the left coronary leaflet using a pair of scissors. The collagen architecture appears uniform with parallel running fibres.

The calcific tear observed in the right coronary leaflet is illustrated in Figure 5. Scanning electron microscopy of the ventricular surface (indicated as Kant B) of this tear showed smooth elevated areas where calcium deposits were located. The fibrous surface (indicated as Kant A) was rougher and elevations due to calcium were not obvious. The edges of the tear were irregular. At higher magnification, both large and small calcific deposits were observed with complete destruction of the collagen architecture. In some areas the extracellular matrix surrounding these crystals was completely absent. Along the edges of the tear where collagen bundles were seen, these were more clumped than cordlike and disrupted. The entire surface appeared to be covered with thin, thread-like fibrin.

The perforations in the left coronary (p1 and p2) and noncoronary leaflets (p3) are shown in Figure 6. Two lesions were found in the central portion of the leaflet. The edges of one of the more elongated of these lesions appeared to be pushed up into the outflow surface of the leaflet. Both lesions appeared as defects in the leaflet and were irregular around the edges. At low magnification collagen bundles appeared severed and discontinuous. At higher magnification, the collagen fibers were coarse and formed thickened bundles but the parallel structure of the fibers could still be discerned. These bundles were covered by a thin fibrin deposit. At still higher magnifications, at the edges, the lesions showed frayed and abruptly terminating collagen fibers with a deposit of fibrin on the surface. The intervening spaces between collagen bundles were not completely filled with fibrin. Away from the edges, where the inflow surface of the leaflet was visible, the latter appeared smooth at low magnification.

Figure 7 shows scanning electron micrographs of the perforation (p3) located at the base of the noncoronary leaflet. The edges of this lesion were also irregular. Even though at lower magnification the parallel nature of the collagen architecture could be seen, at the edges collagen bundles were frayed and abruptly terminated with a loss of preferential orientation. In the middle of this lesion we observed what appeared to be a bulging of the edge of the perforation into the outflow side of the cusp, located in the area of specimen 1938 in Figure 7. The surface of this lesion also appeared to be covered by a thin layer of fibrin.

**DISCUSSION**

This report presents the topographic characteristics of 2 types of lesions in the same valve. The most striking difference between the calcific tear and catheter perforations is the presence of calcium in the former. This finding was consistent with the low concentration of calcium seen in the body of the leaflet. Furthermore, these lesions differed in their location. The calcific tear was located in the right coronary leaflet toward one of the commissures, while the catheter perforations were located more in the body.

**FIGURE 6** Scanning electron micrograph of 2 tears, p1 and p2, in the left coronary leaflet. The numbers indicate areas of the tear that were scanned in more detail. (A, 1913) Edge of p1. Note the short, frayed collagen bundles wirhout the formation of thickened cords. Note also the absence of calcific crystals. (B, 1914) Note the flattened and coarser bundles of collagen with a deposit of fibrin on the surface. (C, 1916) Edge of p2. Note the frayed bundles of collagen, but a retention of the parallel structure (arrowhead). Note the short and severed appearance of the bundles also in this perforation. (D, 1917) At this location the collagen bundles appear to form thicker chords but the parallel architecture is still noticeable. Note again the broken appearance of the bundles.
FIGURE 7  (A, 1936) Overview of the tear p2 at the base of the noncoronary leaflet. The numbers indicate areas that were scanned in more detail. Note the flag-like protrusion into the outflow surface of the valve (arrow). (B, 1937) Note the disrupted collagen bundles with apparently no preferential direction. Note also the fibrin deposit on these bundles and the absence of calcific deposits. (C, 1938) A higher magnification of an area near to 1937; note the fibrin deposit and the parallel collagen bundles. (D, 1939) Note the irregularity of the lesion and the broken collagen bundles. (E, 1940) A low-magnification view of this lesion from the inflow side showing characteristics similar to (D, 1939).
of the leaflet. Preferential calcification of porcine bioprothetic valves has been reported and is a generally accepted observation [12]. Ishihara and co-workers [13] classified noncalcific cuspal tears into 4 types based on morphology and localization: lesions that involved the free edge of the leaflet were classified as type I, those in the central portion of the leaflet as type II, and those at the base of the leaflet type III. Type IV lesions were small pinhole and rounded perforations, which were usually multiple and centrally located. Based on this classification, the calcific tear in our specimen can be classified as a type I lesion. The catheter perforations described in this report, although located at the positions ascribed to type II and type III lesions, differed morphologically from lesions due to mechanical tissue fatigue that were observed by the latter authors in glutaraldehyde-treated porcine valves. In valves tested by accelerated mechanical fatigue, the authors described type II lesions resulting from mechanical fatigue as disruption of collagen resulting in the separation of the parallel collagen bundles from each other. The latter is in stark contrast to the perforations described here in which the collagen bundles were not separated but completely transsected.

In contrast to the calcific tear in which the collagen architecture was completely disrupted or absent, the parallel nature of the collagen bundles was still discernable in the lesions caused by catheter perforation. Bundles of collagen appeared clumped at the edges of the calcific tear, while they appeared more frayed and as coarse cords along the edges of the catheter perforation.

A further characteristic of the bundles of collagen at the edges of the perforations is the apparent lack of preferential direction. The latter observation could be due to the multidirectional breakage of collagen due to the blunt trauma of the catheter as opposed to an incisional lesion. The bulging of the edges of the perforations observed and shown in Figure 7 might be an artifact resulting from the direction from which the catheter approached the leaflet. The deposition of fibrinous material observed in all lesions is not unusual and has been reported to occur within 24 h in implanted valves [2].

In conclusion, ablation catheter perforations, a calcific tear and incisional lesion in porcine aortic valve leaflets are morphologically different in the same aortic valve. This report describes for the first time the topography of ablation catheter perforations and a calcific tear in the same porcine bioprosthetic aortic valve explanted after 12 years and forms a basis for comparison in evaluating lesions in explanted valves by scanning electron microscopy.

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Chapter 3
Hydrodynamic Evaluation of Kangaroo Aortic Valve Matrices for Tissue Valve Engineering

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Hydrodynamic Evaluation of Kangaroo Aortic Valve Matrices for Tissue Valve Engineering

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Abstract: We evaluated the hydrodynamic performance of kangaroo aortic valve matrices (KMs) (19, 21, and 23 mm), as potential scaffolds in tissue valve engineering using a pulsatile left heart model at low and high cardiac outputs (COs) and heart rates (HRs) of 60 and 90 beats/min. Data were measured in two samples of each type, pooled in two CO levels (2.1 ± 0.7 and 4.2 ± 0.6 L/min; mean ± standard errors on the mean), and analyzed using analysis of variance with CO level, HR, and valve type as fixed factors and compared to similar porcine matrices (PMs). Transvalvular pressure gradient ($\Delta$P) was a function of HR ($P < 0.001$) and CO ($P < 0.001$) but not of valve type ($P = 0.39$). $\Delta$P was consistently lower in KMs but not significantly different from PMs. The effective orifice area and performance index of kangaroo matrices was statistically larger for all sizes at both COs and HRs. Key Words: Kangaroo aortic valve matrices—Porcine aortic valve matrices—Hydrodynamics of heart valve matrices—Tissue engineering.

Currently, two types of prosthetic heart valves are available to cardiac surgeons, namely, bioprosthetic valves made from biological material and mechanical valves made from nonbiological material. Both types of devices are associated with significant limitations including a need for anticoagulation, infection, and degeneration (1–6).

A tissue engineered living valve would replenish its extracellular matrix, avoid anticoagulation, and theoretically offer the possibility of growth after implantation.

One approach to tissue valve engineering involves seeding living cells onto an acellular biological matrix in vitro in order to produce a living construct before implantation. Acellular porcine aortic valves have been used by several groups as a biological scaffold onto which autologous cells are seeded (7–11). Once implanted, the seeded cells are expected to replenish the matrix over time using the initial scaffold as a template.

Kangaroos are upright and more similar in posture to humans compared to pigs. Their aortic valves are morphologically and histologically different from porcine aortic valves (12). In particular, kangaroo aortic valves have thinner leaflets with a more compact collagenous matrix compared to porcine aortic valves and unlike porcine aortic valves, have no muscular shelf in the right coronary cusp (12–14). Such a muscular shelf, in addition to possibly obstructing flow, also necessitates muscular decellularization when preparing acellular biological scaffolds. Its absence in the kangaroo valve avoids both possible obstruction with hydrodynamic consequences as well as the need for muscular decellularization. Indeed, Weinhold and coworkers, who implanted whole kangaroo aortic valves in the tricuspid position of sheep, reported superior hemodynamic performance of kangaroo aortic valves compared to porcine ones (14).

A major challenge in tissue valve engineering is sufficient and satisfactory invasion of scaffolds by the repopulating cells. Collagen is a proinvasive substrate for mesenchymal cells (15,16), and the compact collagenous matrix of kangaroo aortic valve leaflets could be an advantage in cellular repopulation while requiring fewer cell for total repopulation

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compared to thicker leaflets. In addition, kangaroo aortic valves have been reported to have at least a comparable if not lower calcification potential compared to porcine aortic valves (12-14). Consequently, the latter reports, we have started investigating the kangaroo aortic valve matrix (KVs) for potential use in tissue valve engineering.

Transvalvular pressure gradients (APs) across cardiac valve prostheses have been identified as one of the most important performance measures in valve replacement surgery. In the aortic position, transvalvular gradients have been postulated to influence both bioprosthetic valve durability by its mechanical tissue effects and patient survival by its modulation of left ventricular hypertrophy (17,18). As a prerequisite for their use in tissue engineering, this study evaluated the hydrodynamics of kangaroo aortic valve matrices (KMs) and compared them to similarly sized porcine aortic valve matrices (PMs).

Cell lysis followed by enzymatic digestion and detergent extraction is commonly used to prepare acellular biological matrices (19,20) and for purposes of this study, matrices were prepared using a patented detergent-enzymatic protocol, which has been successfully used and reported in the literature (21,22).

**MATERIALS AND METHODS**

**Matrix procurement**

The porcine valves used in this study were obtained from the experimental slaughterhouse of the Department of Animal Production, Ghent University. The genotype of all animals was a cross between a hybrid sow and Pietrain boar (BLG; Belgian Land Race). Immediately after sacrifice, hearts were retrieved and placed on wet ice for transportation to the Laboratory of Experimental Cardiac Surgery, University Hospital Ghent. Aortic valves were dissected free as 4-cm-long conduits with a 2-mm rim of myocardium and stored in a cold preserving solution of hypotonic saline. The aortic valves were obtained from Eastern gray kangaroos (*Macropus giganteus*) under license in Queensland, Australia. The valves were procured at the Queensland Heart Valve Bank, University of Queensland, under similar conditions.

For all valves, tissues were decellularized using a detergent-enzymatic treatment. Essential tissues were rinsed in hypotonic saline containing phenylmethylsulfonylfluoride (PMSF: 1 μM, Sigma, Bornem, Belgium) and antibiotics (streptomycin: 100 μg/mL; penicillin: 100 μg/mL mixture; Sigma). Cells were then ruptured by subjecting the tissues to alternating treatments in hypotonic Tris buffer (pH 8.0) and hypertonic Triton X solutions (pH 8.0) (Bio-Rad, Eke, Belgium). All solutions were supplemented with PMSF (1 μM), penicillin/streptomycin solution (100 μg/mL, respectively), and butylated hydroxyanisole (50 μM; Sigma). After thorough rinsing, the tissues were subjected to a digestive procedure using an enzymatic solution containing DNaseI, RNaseA, trypsin, and phospholipases A2, C, and D (Sigma). All steps were performed at 4°C.

The annular size was determined by passing an obturator through the annulus of tested valves. Identical sizes of KMs and PMs were tested. The tested sizes were 19, 21, and 23 mm and for each size, two samples were tested in a pulsatile model of the human left heart. Each valve was implanted in a sized-matched silicone tube. The valve was sewn in place distally and proximally using a running 6.0 prolene suture (Ethicon, Somerville, NJ). The coronary arteries were ligated with Ethibond 2.0 suture (Ethicon).

**In vitro model**

The mock loop is an updated and modified version of a model that was originally designed by Verdonck et al. in 1992 (23). The model has been used before to test heart valves (24-26) and with dedicated modifications to allow for testing assist devices (27,28). The pneumatically driven hydraulic 1:1 in vitro model of the human left heart consists of two silicon sacs representing the left atrium and ventricle, respectively, and separated by a commercially available bioprosthetic valve in mitral position. Inflow in the model is generated through a reservoir (representing the lungs) yielding constant filling pressures. The silicon rubber tube containing the valve matrix under study is connected to the outflow section of the ventricle, such that the valve functionally operates as an aortic valve. At its distal end, the silicon tube is connected to an air chamber (a windkessel) and a hydraulic resistance, mimicking total arterial compliance and systemic vascular resistance, respectively.

The atrial and ventricular silicon sacs are mounted in a Plexiglas chamber filled with water, communicating with vertically mounted cylinders to which pressurized air is delivered (see Fig. 1). For these experiments, only the ventricle was actively controlled. Increasing the pneumatic pressure leads to a volume reduction of the ventricular sac, and ejection through the aortic valve. This emptying leads to a decrease of the fluid level in the vertical cylinder, where the water level is continuously monitored using an ultrasonic water level detector. The circulating fluid within the model was a blood analog consisting of a mixture of 40% glycercine and 60% water with an estimated
dynamic viscosity of 3 mPa at room temperature. The electronic part of the model allows independent regulation and monitoring of left ventricular pressure and volume throughout the cardiac cycle for different heart rates (HRRs). To avoid any alterations in the tissue and its mechanics due to the circulating fluid, the testing of each matrix was completed within 1 h to avoid any adverse effects of the circulating fluid on the valvular tissue. The registered hydrodynamic parameters showed no difference at the end of testing compared to those at the beginning of the testing period, indicating no significant alteration in the hydrodynamic function of the device during the testing time. Similar observations were reported by Chandran and Khalighi, who tested bioprosthetic valves using a similar glycerin solution (29).

Measurment protocol and derived indices

In addition to a pressure sensor directly measuring intraventricular pressure, short, rigid 6F catheters were placed 1 cm proximal and distal to the aortic valve, and connected to piezoelectric pressure measuring devices (Becton Dickinson, Boston, MA, U.S.A.). Pressures were continuously recorded. Stroke volume (SV) was measured as the difference between end-diastolic and end-systolic water level volume in the vertical cylinder, multiplied with the cross-sectional area of the cylinder. Cardiac output (CO) was obtained as the product of HR and SV. The experiments were performed with the pulse duplicator system operating at 60 and 90 beats/min, respectively. Ventricular pressure and/or systemic vascular resistance were varied in order to generate regimes with CO in the range of 1–6 L/min.

The instantaneous pressure difference over the aortic valve was obtained as the difference between proximal and distal pressures (Fig. 2). The mean pressure difference (ΔP, in millimeters of mercury) during forward flow was calculated, with the period of forward flow (ΔTfin) designated by the period during which a positive pressure difference existed (Fig. 2).

Effective orifice area (EOA) is an index of the efficiency of valvular opening and an indication of the degree of obstruction to blood flow by the aortic valve (30).

The EOA was calculated as $EOA = \frac{Q}{51.6\sqrt{\Delta P}}$, where $Q$ is the mean forward flow obtained as $SV/\Delta T_{fin}$ (in milliliter per second) (31).

Finally, performance index (PI) was derived as $PI = EOA/\text{theoretical orifice area} (%)$. The theoretical orifice area was $\pi r^2$, with $r$ being the radius of the valve (32).

The EOA can be normalized to valve size by calculating the PI of the valves tested. The PI is another measure of valvular obstruction to flow and is derived by the formula $PI = EOA/\text{geometrical orifice area} (%)$. The geometrical orifice area is represented by $\pi r^2$, with $r$ being the radius of the valve calculated from the measured diameter (32).

Statistics

Data were first analyzed by plotting $\Delta P$ as a function of $Q$. Subsequently, the data were categorized in

[FIG. 1. Pneumatically driven hydraulic 1:1 mock model of the human left heart. Top right: cardiac chambers with the left atrium and ventricle; right: test section with sewn KM in place. Flow through the valve is from right to left. At the bottom, pictures of typical PMs and KMs are shown in inset.]
two CO levels (low: CO ≤3 L/min and high: CO ≥3 L/min). We then applied analysis of variance (ANOVA) with CO level (low/high), valve sizes (19, 21, and 23 mm), HR (60 and 90 beats/min), and valve origin (porcine and kangaroo) as independent factors and tested in how far these factors determined the values of ΔP, EOA, and PI. The significance level was set to a P value of 0.05. All analyses were carried out in SPSS 12.0 (SPSS, Inc., Chicago, IL, U.S.A.).

RESULTS

The calculated values for ΔP and EOA are shown in Table 1A–C. Low CO was 2.1 ± 0.7 L/min, while

### Table 1. ΔP, EOA, and PI for 19-, 21-, and 23-mm PMs and KMs at 60 and 90 beats/min

<table>
<thead>
<tr>
<th>CO</th>
<th>HR</th>
<th>Species</th>
<th>ΔP</th>
<th>P value</th>
<th>EOA</th>
<th>P value</th>
<th>PI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. ΔP, EOA, and PI for 19-mm valve matrix at HRs of 60 and 90 beats/min</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>60</td>
<td>KM</td>
<td>3.04 ± 0.13</td>
<td>NS</td>
<td>1.58 ± 0.20</td>
<td>P &lt; 0.05</td>
<td>0.570 ± 0.008</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PM</td>
<td>3.35 ± 0.13</td>
<td></td>
<td>1.46 ± 0.26</td>
<td></td>
<td>0.506 ± 0.009</td>
<td></td>
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<tr>
<td></td>
<td>90</td>
<td>KM</td>
<td>1.92 ± 0.14</td>
<td>NS</td>
<td>1.46 ± 0.30</td>
<td>P &lt; 0.05</td>
<td>0.537 ± 0.009</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PM</td>
<td>2.23 ± 0.14</td>
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<td>1.34 ± 0.26</td>
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<td>0.473 ± 0.009</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>60</td>
<td>KM</td>
<td>6.50 ± 0.14</td>
<td>NS</td>
<td>1.98 ± 0.27</td>
<td>P &lt; 0.05</td>
<td>0.695 ± 0.009</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PM</td>
<td>6.81 ± 0.13</td>
<td></td>
<td>1.86 ± 0.28</td>
<td></td>
<td>0.631 ± 0.008</td>
<td></td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>KM</td>
<td>5.37 ± 0.13</td>
<td>NS</td>
<td>1.86 ± 0.28</td>
<td>P &lt; 0.05</td>
<td>0.662 ± 0.008</td>
<td>P &lt; 0.05</td>
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<tr>
<td></td>
<td></td>
<td>PM</td>
<td>5.68 ± 0.13</td>
<td></td>
<td>1.74 ± 0.29</td>
<td></td>
<td>0.598 ± 0.008</td>
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<tr>
<td>B. ΔP and EOA for 21-mm valve matrix at HRs of 60 and 90 beats/min</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Low</td>
<td>60</td>
<td>KM</td>
<td>3.16 ± 0.15</td>
<td>NS</td>
<td>1.71 ± 0.27</td>
<td>P &lt; 0.05</td>
<td>0.559 ± 0.010</td>
<td>P &lt; 0.05</td>
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<tr>
<td></td>
<td></td>
<td>PM</td>
<td>3.49 ± 0.15</td>
<td></td>
<td>1.59 ± 0.29</td>
<td></td>
<td>0.495 ± 0.010</td>
<td></td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>KM</td>
<td>2.65 ± 0.15</td>
<td>NS</td>
<td>1.59 ± 0.30</td>
<td>P &lt; 0.05</td>
<td>0.537 ± 0.010</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PM</td>
<td>2.36 ± 0.16</td>
<td></td>
<td>1.47 ± 0.30</td>
<td></td>
<td>0.462 ± 0.010</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>60</td>
<td>KM</td>
<td>6.63 ± 0.16</td>
<td>NS</td>
<td>2.11 ± 0.30</td>
<td>P &lt; 0.05</td>
<td>0.684 ± 0.010</td>
<td>P &lt; 0.05</td>
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<tr>
<td></td>
<td></td>
<td>PM</td>
<td>6.94 ± 0.15</td>
<td></td>
<td>1.99 ± 0.28</td>
<td></td>
<td>0.620 ± 0.010</td>
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<tr>
<td></td>
<td>90</td>
<td>KM</td>
<td>5.51 ± 0.15</td>
<td>NS</td>
<td>1.99 ± 0.28</td>
<td>P &lt; 0.05</td>
<td>0.651 ± 0.009</td>
<td>P &lt; 0.05</td>
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<tr>
<td></td>
<td></td>
<td>PM</td>
<td>5.81 ± 0.14</td>
<td></td>
<td>1.87 ± 0.29</td>
<td></td>
<td>0.587 ± 0.009</td>
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<tr>
<td>C. ΔP, EOA, and PI for 23-mm valve matrix at HRs of 60 and 90 beats/minute</td>
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<tr>
<td>Low</td>
<td>60</td>
<td>KM</td>
<td>2.65 ± 0.15</td>
<td>NS</td>
<td>1.79 ± 0.27</td>
<td>P &lt; 0.05</td>
<td>0.441 ± 0.010</td>
<td>P &lt; 0.05</td>
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<tr>
<td></td>
<td></td>
<td>PM</td>
<td>2.96 ± 0.14</td>
<td></td>
<td>1.66 ± 0.29</td>
<td></td>
<td>0.377 ± 0.009</td>
<td></td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>KM</td>
<td>1.57 ± 0.16</td>
<td>NS</td>
<td>1.67 ± 0.30</td>
<td>P &lt; 0.05</td>
<td>0.408 ± 0.010</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PM</td>
<td>1.83 ± 0.14</td>
<td></td>
<td>1.54 ± 0.30</td>
<td></td>
<td>0.344 ± 0.009</td>
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<tr>
<td>High</td>
<td>60</td>
<td>KM</td>
<td>6.30 ± 0.16</td>
<td>NS</td>
<td>2.18 ± 0.30</td>
<td>P &lt; 0.05</td>
<td>0.565 ± 0.010</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PM</td>
<td>6.41 ± 0.14</td>
<td></td>
<td>2.06 ± 0.28</td>
<td></td>
<td>0.501 ± 0.009</td>
<td></td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>KM</td>
<td>4.98 ± 0.16</td>
<td>NS</td>
<td>2.06 ± 0.28</td>
<td>P &lt; 0.05</td>
<td>0.532 ± 0.010</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PM</td>
<td>5.29 ± 0.14</td>
<td></td>
<td>1.94 ± 0.29</td>
<td></td>
<td>0.406 ± 0.009</td>
<td></td>
</tr>
</tbody>
</table>

All values listed in the table are mean ± standard errors on the mean.  
"Low" CO level was 2.1 ± 0.7 L/min, while "High" CO was 4.2 ± 0.6 L/min; ΔP in millimeters of mercury; EOA in cubic centimeter; HR in beats per minute.  
NS, not significant.
high CO was 4.2 ± 0.6 L/min. The results of the regression analysis or ΔP versus CO at 60 and 90 beats/min are shown in Fig. 3. The pressure gradient (ΔP) as a function of valve size is shown in Fig. 4.

Statistical analysis with CO as a categorical variable revealed that ΔP was a function of HR (P < 0.001) and CO level (P < 0.001) but not of matrix type (P = 0.01). The difference in ΔP between KMs and PMs was in the order of 0.3 mm Hg, a difference that was, however, too small to be confirmed in the post hoc analysis when both types of matrices are compared for a given size, HR, and CO level (see Table 1). Both EOA and PI were a function of the same four tested parameters, with all parameters being highly significant (all P < 0.05) between KMs and PMs for all tested cases, with higher EOA (and PI) for the KMs (90 and 60 beats/min).

DISCUSSION

It is widely acknowledged that the performance of prosthetic heart valves is critically related to their fluid dynamic properties (32). Left ventricular pressure is a major determinant of myocardial oxygen consumption (33). The pressure developed in the left ventricle during the ejection phase of cardiac systole is directly proportional to the transvalvular gradient of the aortic valve with higher gradients necessitating higher pressures. The transvalvular pressure gradient (ΔP) across KMs compared favorably to that of PMs. There was no significant difference in the transvalvular gradients among 19-, 21-, and 23-mm KMs and PMs. Interestingly, the gradients across KMs were consistently lower than those measured for PMs.

A larger EOA corresponds to a smaller ΔP and therefore lower ventricular energy expenditure. KMs showed significantly higher EOAs compared to similarly sized PMs, and suggested less obstructive flow. These observations are in keeping with earlier reports of the in vivo hemodynamic evaluation of kangaroo whole valves in the sheep model (14). Weinhold and coworkers calculated and reported consistently higher EOAs in kangaroo aortic valves even after prolonged implantation in the sheep. Kangaroo aortic valves are morphologically different from porcine ones, and these morphological differences may contribute to the observed differences in EOA. In contrast to porcine valves, kangaroo valves do not have a muscular shelf in the right coronary cusp, which may contribute to the larger EOAs observed in KMs (14).

The PI in KMs was consistently larger than that in PMs and is consistent with the larger EOAs observed.

It should be noted that in this in vitro setting with rather short tube segments connected to the downstream air chamber and resistance, nonphysiological wave reflections arise and the pressure waveforms presented with some nonphysiological fluctuations, especially during diastole. Nevertheless, we only used data measured during ejection, and although some oscillations do exist during ejection and have an effect on the time course of pressure and pressure difference, the effect on the outcome is expected to be small, as we only work with time-averaged pressure differences.

Finally, it should be acknowledged that the study is subject to some methodological limitations. In particular, flow waveforms were found difficult to measure close to the valve with reasonable accuracy and signal-to-noise ratio in our setting with the short aortic roots sewn in the silicon tube. This was not a problem in the assessment of SV, which was obtained in an indirect way (see Methods section), but it would certainly have allowed us to more accurately define the period of forward flow, during which mean pressure gradients were calculated. We now assumed that the start and end of forward flow coincided with the zero crossover of the transvalvular pressure difference, which is not necessarily the case due to inertial effects. We also performed all pressure measurements with short catheters connected to fluid-filled pressure transducers. The absolute accuracy of these devices on an individual measurement is, at best, estimated to be 0.5 mm Hg, probably closer to 1 mm Hg. The fact that we could yet demonstrate a significant pressure difference of less than 0.5 mm Hg is due to the ANOVA approach, which allowed including all the measurements from the different valves.

CONCLUSION

In conclusion: (i) both KMs and PMs showed an increase in ΔP and EOA with increasing CO; (ii) although for all sizes tested, there was no significant difference in the mean gradient at either low or high COs, and at 60 or 90 beats/min, KMs showed consistently lower transvalvular gradients; (iii) the EOA and PI of KMs were statistically larger for all three sizes of valves at both low and high CO and at both 60 and 90 beats/min; and (iv) while this study examined only the hydrodynamic properties of KMs and PMs, the results are encouraging for further investigations into the use of KMs in tissue valve engineering.

Acknowledgments: This research was partly funded by the fund for scientific research—Flanders.
FIG. 3. Illustration of the "raw data" showing $\Delta P$ as a function of CO for all tested valves. Closed symbols represent KMs and open symbols represent PMs. The solid and broken lines are the (quadratic) regression lines through the kangaroo and porcine data, respectively (data pooled from two samples). The regression lines were added only to allow for a better visual discrimination between the data of the two valve types. The coefficients of the nonlinear regression were not used in the analysis.
Belgium (FWO-Vlaanderen; Krediet aan Navorsers). The authors wish to acknowledge the technical and logistical support of Elke Leccoq, Technician, Laboratory of Protein Engineering, University of Ghent, Mieke Olieslagers, Research Nurse, Laboratory of Experimental Cardiac Surgery, University Hospital Ghent, and Jurgen Deviche, Technician, Hydraulics Laboratory, University of Ghent.

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morphology, tensile strength and calcification potential.


Part II

Storage of Matrices by Cryopreservation:
Effects on the Biomechanical Properties of Xenogenic Matrices

Clinical use of a tissue engineered valve, when developed using current approaches to tissue valve engineering will have to take place within the context of the clinical realities of valve replacement surgery and the limitations of tissue transplantation, including the logistics of supply, preparation before surgery and availability of appropriate sizes. At present methods used in the preparation of matrices can take as long as one week [1] and such procedures while they can be planned before surgery are not an option in semi-urgent or urgent cases in the clinical setting. The decellularization protocol employed by us and described in chapter I, is five days in duration. It became clear to us that the practicality of such procedures might be problematic in a clinical setting and that having readily available, preserved matrices would be a more practical option. Consequently, we investigated the feasibility of cryopreserving matrices.

Although cryopreservation has been used for decades to preserve homografts [2] it is known to result in tissue damage with necrotic changes, disrupted cellular ultrastructure, damage to the extra-cellular matrix and loss of proteoglycans. However the mechanical properties appeared to be preserved despite the histological changes [3,4,5]. The studies in this chapter were consequently performed for two main reasons.

Firstly, the effects of cryopreservation on xenogenic matrices have not been reported previously and to check these effects on the matrices prepared using our protocol, we examined proteoglycan and collagen content as well as the mechanical strength of porcine matrices before and after cryopreservation. Are they still strong enough after cryopreservation?

Secondly, the removal of cells and at least some of the soluble proteins from the tissue by decellularization must result in some alteration in their properties. Nevertheless, mechanical studies performed on a-cellular porcine matrices using burst pressure and uni-axial tests suggest that the material properties of the extra-cellular
matrices approach those of native valves and hence are preserved [6-8]. Despite these observations, there is no a priori reason why this should be so and these same studies report histological findings in matrices that indicate high porosity and locally collapsed microstructure in matrices. In this study we also evaluated the effects of the decellularization process used by us on porcine matrices by comparing them to fresh tissue since such treatment may also alter tissue material properties.

References


Chapter 4
Readily Available Porcine Aortic Valve Matrices For Use in Tissue valve Engineering. Is Cryopreservation an Option?

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Abstract

The clinical use of acellular biological valves as scaffolds in tissue valve engineering would require them to be readily available. This study examines the feasibility of cryopreserving porcine aortic valve matrices for use in tissue valve engineering. Matrices prepared using an enzymatic-detergent decellularization protocol were examined before and after cryopreservation. The biochemical status of tissues were evaluated by collagen and uronic acid (proteoglycan) determination and their mechanical properties were determined using a burst test. The histological and ultrastructural properties were evaluated by light and electron microscopy.

Cryopreservation did not significantly affect the collagen and uronic acid content of aortic leaflet matrices. Histological and ultrastructural sections, however, confirmed extensive disruption of the extracellular collagen matrix and inter-fibrillar proteoglycan associations following cryopreservation. Although neither the breakage force nor the maximum force at failure was significantly different in matrices before and after cryopreservation, the strain observed in matrices was significantly higher after cryopreservation.

To our knowledge this is the first study to investigate the effects of cryopreservation on aortic valve matrices. Cryopreservation did not significantly alter the biochemical properties of porcine aortic valve matrices. Nevertheless, cryopreservation had significant adverse effects on the structural and mechanical properties of matrices. Cryopreserved matrices showed significantly higher strain when stressed compared to non cryopreserved matrices. While, theoretically matrices are only expected to be functional for a limited time until regenerated in vivo, further mechanical testing is necessary to evaluate the effects of these changes on the durability of porcine aortic valve matrices for use in tissue valve engineering.

Key Words: Porcine Aortic Valve Matrices, Cryopreservation, Biological Valve Matrices, Tissue Engineering Matrices.
1. Introduction

Tissue engineered cardiac valves offer a means of overcoming the many limitations of contemporary cardiac valve prostheses. Currently available prosthetic cardiac valves are of two types; (a) bioprosthetic valves, made from either animal or human tissue and (b) mechanical valves made from non biological material. Mechanical prosthetic valves, though very durable, require life long anticoagulation with its associated risks [13, 46, 5, 26]. Bioprosthetic valves do not require life long anticoagulation but their lack of living cells to replenish and maintain their tissue renders them prone to early deterioration [15, 17]. A tissue engineered living valve, would replenish its extracellular matrix, avoid anticoagulation and theoretically offer the possibility of growth after implantation.

One approach to tissue valve engineering involves seeding living cells onto an acellular biological matrix in vitro in order to produce a living construct before implantation. Acellular porcine aortic valves have been used by several groups as a biological scaffold onto which living cells are seeded [42, 48, 43, 30, 6]. The clinical use of such valves would require them to be available on short notice. However, before such a valve can be implanted into a particular patient, currently available technology would require the procurement of a suitably sized valve from an appropriately sized animal and its subsequent decellularisation and quality control before being repopulated with living cells. The efficiency of such a process would be greatly enhanced if quality controlled matrices of several sizes were readily available and thereby alleviate the need of matrix production each time a valve is required.

It is essential that any method employed to preserve matrices and to make them available ‘off the shelf’ do not significantly alter their properties. Several methods including glutaraldehyde fixation, preservation in antibiotic solutions and cryopreservation are currently used to preserve heart valves. Cryopreservation has been used for several years to preserve human allograft valves for use in aortic valve replacement [28]. The aims of this study were to investigate the effects of cryopreservation on the structural and biochemical properties of porcine aortic valves matrices, as a means of having readily available, stored matrices for use in tissue valve engineering.

Aortic valvular grafts are usually implanted as conduits and are comprised of the aortic leaflets and associated aortic wall. While other studies in our laboratory are focused on the aortic wall, in this study we report only the effects of cryopreservation on aortic leaflet matrices. The extra cellular leaflet matrix contain a variety of structures and performs a variety of functions including mechanical support and physical strength [34,25,37]. In addition it influences cellular adhesion, migration...
and gene expression following cellular contact [1,40]. Consequently the structural and functional integrity of leaflet matrices are crucial to satisfactory valvular performance.

At present, there is no standardized method of preparing valvular matrices for tissue valve engineering. Cell lysis followed by enzymatic digestion and detergent extraction is commonly used to prepare acellular biological matrices [43] and for purposes of this study, matrices were prepared using a patented detergent-enzymatic protocol which has been successfully used and reported in the literature [45, 47]. Biochemical properties were evaluated by determination of the collagen and proteoglycan content in leaflets and structural properties by a standard burst test.

2. Methods

2.1 Valve Procurement

Porcine valves used in this study were obtained from the experimental slaughter house of the University of Ghent, Department of Animal Production. Animals were seven month old commercial slaughter pigs weighing approximately 110 kg each. The genotype of all animals was a cross between a hybrid sow and piétrain boar (BL: Belgian Land Race). The mean annular diameter of valves used was 23 mm. Immediately after slaughter, hearts were retrieved and placed on wet ice for transportation to the Laboratory of Experimental Cardiac Surgery, University Hospital Ghent. Aortic valves were dissected free as four centimetres long conduits with a two millimetres rim of myocardium.

Valves were procured on nine occasions depending on availability. Valves from each procurement day were used to determine one or more parameters. As such for each parameter valves from two or more procurement days were used. Although procurement on different days was out of logistical necessity, it allowed minimization of possible batch effects. The variability in the number of valves available on a particular day meant that the number of valves used to determine each parameter was not always the same and in particular we had more valves available for testing as matrices after cryopreservation.

Valves were processed whole and leaflets excised for analysis. Two hundred and twenty six porcine aortic valve leaflets were studied. Sixty four leaflets were studied fresh (collagen determination n=11, Uronic acid determination n=15, and mechanical testing n=38), 98 were studied after decellularisation (collagen determination n=15, uronic acid determination n= 15, and mechanical testing n= 68) and 64 matri-
ces after cryopreservation (collagen determination n=13, uronic acid determination n=20, and mechanical testing n=31).

Five additional valves were each divided into three parts, each comprising one leaflet and its adjacent aortic wall, which were used for ultrastructural and histological analysis. One part of each valve was examined fresh, while the other two were decellularised to produce matrices, one of which was examined before and the other after cryopreservation.

### 2.2 Decellularisation

Matrices were obtained using a detergent-enzymatic treatment as described by Wilson and co-workers [45] and Zeltinger and co-workers [47]. Essentially tissues were rinsed in hypotonic saline containing phenyl-methylsulfonylfluoride (PMSF: 1µM, SIGMA, Bornem, Belgium) and antibiotics (streptomycin: 100µl/l; penicillin: 100µl/l mixture; SIGMA, Bornem, Belgium). Cells were then ruptured by subjecting the tissues to alternating treatments in hypotonic Tris-buffer (pH 8.0) and hypertonic Triton X solution (pH 8.0) (Biorad, Eke, Belgium). All solutions were supplemented with PMSF (1µM), penicillin/streptomycin solution (100µl/l respectively) and 50 µM butylated hydroxyanisole (SIGMA, Bornem, Belgium). After thorough rinsing, tissues were subjected to a digestive procedure using an enzymatic solution containing Dnase1, RnaseA, trypsin and phospholipases A2, C, and D (SIGMA, Bornem, Belgium). To ensure complete digestion we employed two enzymatic treatments of 45 minutes each. Tissues were then washed for 24 hours in a magnesium and calcium free chelating solution. All steps were performed at 4°C.

### 2.3 Cryopreservation

Valves were cryopreserved according to the protocol of the tissue bank of the University Hospital, Ghent. Valves were placed into hanks balanced salt solution (HBBS, Gibco, Belgium) supplemented with antibiotics and 15 % dimethyl sulfoxide (Me₂SO) (Merck, Darmstadt, Germany). Specimens were then cryopreserved by freezing at a controlled rate of 1° C per minute down to a temperature of –80°C, followed by a decrease of 10°C per minute to a temperature of –100°C. Valves were subsequently stored in the vapour phase of liquid nitrogen at –196°C for a minimum of two weeks before they were studied. Cryopreserved valves were gently thawed, first in a 37°C warm water bath and rinsed alternately for four minutes in each of three solutions of 250 ml of hanks balanced salt solution containing 26 ml, 13 ml and 6 ml DMSO respectively at room temperature, before final rinsing in
HBSS alone. Valves were inspected and found to be free from cracks or other gross damage after thawing.

2.4 **Collagen Determination**

Total collagen was determined according to the protocol of The International Standards Organisation; ISO/DIS 3496.2 [21]. Essentially, dried (30 minutes at 103 °C) porcine leaflets were hydrolysed using 6M HCl at 110°C for 24 hours. The released hydroxyproline was determined after oxidation with chloramine-T, resulting in formation of pyrrole-2-carboxylic acid and pyrrole. Pyrrole was determined using a colour reaction with Ehrlich’s Reagent (p-dimethylaminobenzaldehyde) in perchloric acid and isopropanol to give dipyrryl-phenylmethene salt and ms-tetra-(4-dimethylaminophenyl)-porphin . The latter was read spectrophotometrically at 558 nm and the percentage of collagen was calculated based on a standard curve using hydroxyproline solutions of known concentration ranging between 0.5 and 4 µg/ml subjected to the same colour reaction and using a conversion factor of 8.

2.5 **Histology and Ultrastructure**

2.5.1 **Electron Microscopy**

Samples were fixed in phosphate buffered solution of 4% formaldehyde (Merck, Darmstadt, Germany), supplemented with alcian blue to precipitate and preserve the proteoglycans during the dehydration. Specimens were subsequently post fixed with 1% osmium tetroxide (OsO₄) in phosphate buffer (Merck, Darmstadt, Germany) and embedded in epoxy resin. Ultra-thin 60nm sections were cut and examined with a Jeol 1200 EX-II transmission electron microscope at 80 keV (Jeol, Zaventem, Belgium).

2.5.2 **Light Microscopy**

Samples for histology were fixed in 4% phosphate buffered formaldehyde (Merck, Darmstadt, Germany) and embedded in paraffin. Five micron thick sections were cut and stained with hematoxylin/eosin or with alcian blue and viewed by Axiovert S 100 light microscope (Axiovert S 100, Zeiss, Zaventem, Belgium).
2.5.3  

Uronic Acid Determination

Uronic acid content was used as an estimate of the amount of proteoglycan present in matrices based on the methods described by Ahmed et al [1] and Blumenkrantz and Asboe-Hansen [8]. Essentially, after freeze drying, samples were weighed and then hydrolysed with 5ml of 2.5% sulphuric acid (H$_2$SO$_4$) in an autoclave at 121°C for one hour. The hydrolysate was filtered and three aliquots of 0.6 ml each were used for uronic acid determination. To each aliquot 3 ml of tetraborate solution (0.0125M in concentrated H$_2$SO$_4$) was added and solutions were placed at 100°C for five minutes. The colour reaction was developed by adding MHDP (0.15 % 2-hydroxy-bifenyl in 0.15 % NaOH) solution to two aliquots. The third aliquot was used as a blank in which 60 µl 0.5% NaOH was added instead of MHDP. Absorbance was measured at 520 nm. Uronic acid was determined based on a standard curve of glucuronic acid at concentrations ranging from 10 to 150 µg/ml.

2.5.4  

Mechanical Testing

Whole leaflets were mechanically tested using a burst test similar to the standardized burst tests ((ISO 3379 (1976-02), ASTM D 3786, ASTM 3787-01, DN EN 12332-2(2003)) that are commonly used for flat flexible materials [10, 4, 22]. The analysis employed a texture analyser TA500 (TM Lloyd Instruments, UK) interfaced to a computer using the Nexigen 3.0 / Ondio software packages (TM Lloyds Instruments, UK) which allowed electronic registration of data. Tissues were placed over an opening of 7 mm between two metal plate-clamps and secured using two pieces of water proof sandpaper (Nr. P120) to prevent slipping. Force at a cross head speed of 100 mm/minute was applied onto the leaflet until breakage by a polished ball tipped plunger of 4.5 mm diameter (Fig.1). Care was taken to ensure that leaflets completely covered the opening in the plates with the nodule of Arantius in the centre of the opening for consistency. Leaflets were placed flatly onto the plates and kept taught until clamped. All measurements were done at room temperature and tissues were kept moist with isotonic salt solution. The stress/strain curves for all tissue tested were obtained by conversion of the load/extension curves to stress/strain curves as described in Appendix 1.
2.5.5 Statistics

All statistical analysis in this study was performed using student’s ‘t’ test. Differences with a $p$ value <0.05 were taken to be significant. Mean values are expressed with their corresponding standard deviations.

3. Results

3.1 Decellularisation, Histology and Ultrastructure

Fig. 2 (a, b, c) shows representative light microscopic preparations stained with alcian blue of fresh porcine aortic valve leaflets and of matrices before and after cryopreservation. Matrices were almost completely acellular compared to fresh tissue. In all three tissue types a collagenous matrix was observed. Proteoglycan staining by alcian blue was more extensive in fresh tissue compared to both types of matrices. The extent of alcian blue staining of proteoglycans in cryopreserved matrices did not appear to be significantly different from that in non-cryopreserved matrices. Nevertheless, in histological preparations of matrices that were cryopreserved, there were more areas of what appeared to be empty spaces, when compared to matrices that were not cryopreserved.
Figure 2. Representative light microscopic (a, b, c, Magnification X50; Alcian Blue Stain (Proteoglycans stain blue)) and electron microscopic (d, e, f, Magnification X 2500) preparations of fresh porcine aortic valve leaflets and porcine aortic valve matrices before and after cryopreservation. Matrices (b and c) were acellular compared to fresh tissue (a) and showed less staining of proteoglycans. In d, e and f, the electron dense material represent precipitated proteoglycans (arrow head) and collagen is represented by the less electron dense linear structures (arrow).
Representative electron micrographs of all three types of tissue are shown in Fig. 2 (d, e, f). In fresh tissue, thick bundles of transversely and longitudinally sectioned collagen fibers were observed with electron dense precipitates representative of proteoglycans in between. Matrices that were not cryopreserved also showed longitudinally and transversely cut collagen fibers. Unlike the fresh tissue, however, collagen fibers appeared to have a looser association with each other and the extra-collagenous matrix was less electron dense. Electron dense precipitates of proteoglycans could still be seen bridging adjacent collagen fibers. In cryopreserved matrices, the collagen fibers lost most of their striated appearance and indicated extensive disruption and destruction. Fibers were typically less densely packed and appeared loosely associated compared to non-cryopreserved matrices. Electron dense precipitates of proteoglycans, although still present, appeared clumped and unlike fresh tissue and non-cryopreserved matrices did not seem to cross-link adjacent collagen fibers.

3.2 Biochemistry

Table 1 summarises the collagen and uronic acid content of fresh leaflets and of leaflet matrices before and after cryopreservation. The average collagen content in fresh leaflets was 49%. In matrices that were not cryopreserved and cryopreserved matrices, the average collagen content was 63.3% and 60.3% respectively, and were not significantly different (p = 0.485).

Uronic acid content in fresh leaflets was 9.6 µg/mg dry weight. In matrices that were not cryopreserved, and cryopreserved matrices, the uronic acid content was 2.8µg/mg and 3.2 µg/mg dry weight respectively and these values were not significantly different (p=0.540).

Table 1. Collagen content (%) and uronic acid content (µg/mg) expressed on dry material of fresh leaflets and matrices before and after cryopreservation (cryo).

<table>
<thead>
<tr>
<th>Biochemical component</th>
<th>Fresh porcine leaflets</th>
<th>Matrices before cryopreservation</th>
<th>Matrices after cryopreservation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valve leaflet</td>
<td>49.5a (11)</td>
<td>63.3b (15)</td>
<td>60.3b (13)</td>
</tr>
<tr>
<td>Uronic acids (µg/mg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valve leaflet</td>
<td>9.0a (6)</td>
<td>2.8 b (5)</td>
<td>3.2 b (5)</td>
</tr>
</tbody>
</table>

a,b: values having different superscripts differ significantly between columns (p<0.05), ( ) = number of observations.

3.3 Mechanical strength

Fig. 3a illustrates typical load/extension curves obtained for each type of tissue. The typical curve obtained showed an initial extension followed by a transition to a steep
rising phase with a slight reduction in slope before it peaks. The change in slope represents the point at which the tissue started to fail and is referred to as the breakage force. The peak of the curve coincided with perforation of the leaflet and represented the maximum force registered. The Failure phase (FP) of the curve was represented by the duration from the commencement of failure (breakage force) to perforation of the leaflet (Fig. 3b). Figure 4 (a, b, c) shows representative series of measurements obtained for each type of tissue to maximum force. The breakage force was not significantly different in the three types of tissue (Table 2). FP was more abrupt in fresh leaflets and the average maximum force reached was significantly lower than in matrices. Neither FP nor the average maximum force was significantly different in matrices. Representative stress/strain curves for each type of tissue are illustrated in Figure 5 (a, b, c). The strain at maximum force in matrices that were not cryopreserved was not significantly different from that of fresh tissue. In cryopreserved matrices, the strain at both the breakage force and the maximum force was significantly higher compared to non cryopreserved matrices and fresh (Table 3).

**Table 2. Average values of parameters derived from the load/extension curves resulting from the multiaxial test on fresh valve leaflets and valve matrices before and after cryopreservation.**

<table>
<thead>
<tr>
<th>Porcine valve</th>
<th>Fresh</th>
<th>Matrix before cryopreservation</th>
<th>Matrix after cryopreservation</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>38</td>
<td>68</td>
<td>31</td>
</tr>
<tr>
<td>Maximum force (N)</td>
<td>17.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Breakage force (N)</td>
<td>17.5</td>
<td>18.6</td>
<td>19.1</td>
</tr>
<tr>
<td>Failure phase (mm)</td>
<td>0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.43&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b</sup>: values having different superscripts differ significantly between columns (p<0.05), ( ) = standard deviation.

**Table 3. Average values of parameters derived from the stress/strain curves as derived from the load/extension curves resulting from the multiaxial test on fresh valve leaflets and valve matrices before and after cryopreservation.**

<table>
<thead>
<tr>
<th>Fresh Porcine Leaflets</th>
<th>Matrix before cryopreservation</th>
<th>Matrix after cryopreservation</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>38</td>
<td>68</td>
</tr>
<tr>
<td>Strain (%) at</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breakage Force</td>
<td>49.7&lt;sup&gt;a&lt;/sup&gt; (41.1)</td>
<td>54.3&lt;sup&gt;a&lt;/sup&gt; (32.3)</td>
</tr>
<tr>
<td>Maximum Force</td>
<td>52.6&lt;sup&gt;a&lt;/sup&gt; (41.1)</td>
<td>63.2&lt;sup&gt;a&lt;/sup&gt; (36.3)</td>
</tr>
<tr>
<td>Stress (kPa) at</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breakage Force</td>
<td>310.4 (74.1)</td>
<td>322.7 (88.3)</td>
</tr>
<tr>
<td>Maximum Force</td>
<td>310.9 (74.6)</td>
<td>334.1 (82.3)</td>
</tr>
</tbody>
</table>

<sup>a,b</sup>: values having different superscripts differ significantly between columns (p<0.05), ( ) = standard deviation.
Figure 3a. Representative load/extension curves for each type of tissue.

Figure 3b. Illustration of the failure phase (FP) on the load extension curves.
Figures 4 (a,b,c). Representative series of load/extension curves for fresh tissue (a), matrices (b) and cryopreserved matrices (c), shown to maximum force.
Figure 5 (a, b, c): Stress/strain curves as derived from the load/extension curves resulting from the multiaxial test on fresh tissue (a), matrices before cryopreservation (b) and matrices after cryopreservation (c).
4. Discussion

The extracellular matrix of valvular leaflets is comprised of collagen, proteoglycans and elastin organised into three distinct layers, namely the fibrosa, the ventricularis and the spongiosa sandwiched between the latter two [38]. The main structural layer is the fibrosa which is comprised mainly of multidirectional collagen with a smaller amount of elastin [31]. Towards the belly of the leaflet, away from the free edge at the point of force application as in this study, collagen fibers are increasingly multidirectional [31]. The collagen fibers are surrounded by proteoglycans which is also the major component of the spongiosa layer.

There was no significant change in total collagen content nor the amount of proteoglycans in matrices after cryopreservation. (Table 1). Yun Hee and Wolfinbarger [47], who studied the effects of cryopreservation on proteoglycans in aortic tissue, reported no loss of proteoglycans after cryopreservation and our findings in valvular leaflet matrices is in keeping with these observations. The lower proteoglycan content in matrices compared to fresh tissue (p<0.005) is consistent with earlier reports showing a loss of proteoglycans following decellularisation of porcine aortic valves [36]. Collagen was determined as a proportional percentage of tissue dry weight. Decellularization is known to result in a loss of dry weight of fresh aortic valve leaflets [36]. This loss of material content with decellularisation could explain the higher proportional collagen content observed in matrices compared to fresh tissue (p<0.005). After cryopreservation, alcian blue stain did not show a difference in the amount of proteoglycans present in cryopreserved and non cryopreserved matrices and supported the quantitative findings.

The mechanical properties of heart valves, like similar connective tissue material, is determined by a complex of forces including such relations as complementation between elastin and collagen and proteoglycan-fibrillar interactions [7, 41]. The consequences of such interactions results in specific anisotropic properties in tissues such as aortic valve leaflets. In addition, it has also been suggested that heart valves achieve their function partly as a result of a translational movement of the fibrosa and ventricularis relative to each other resulting in an increase in valve surface area during diastole [39]. Such movements involve considerable shear forces within the leaflet and are believed too be facilitated by the proteoglycan rich spongiosa layer between the fibrosa and ventricularis layers. Since this study focused on the properties of the leaflets as a whole, the burst test was preferred to uniaxial or biaxial test as it allowed us to address both the contribution of the multidirectional collagen architecture without decoupling of fibers as is the case when strips of tissue are tested as
in uniaxial tests [11] and the possible translational movements within leaflets (appendices 2 and 3).

The breakage force was not significantly different between the three types of tissues. In fresh tissue, however, the maximum force was significantly higher than in matrices (table 2). The calculated strain was higher in matrices compared to fresh tissue although not significantly different between fresh tissue and matrices that were not cryopreserved (table 3). Cryopreserved matrices, however, exhibited significantly higher strain at both the breakage force and maximum force compared to matrices that were not cryopreserved.

Both a loss of proteoglycan and disruption of collagen fibers may contribute to increased strain as the tissue is stressed to breakage. Inter-fibre proteoglycans can play a significant role in tissue mechanics. When stressed, collagen fibers in aortic leaflets have been shown to undergo extensive fiber rearrangement [32]. Loss of inter-fibrillar proteoglycans could impede fiber realignment and prevent the even redistribution of load in the test material with high strain in areas of higher stress, thereby offering a possible explanation for the higher strain observed in matrices [9]. The latter implies that in fresh tissue with a higher proteoglycan content compared to matrices, a more even distribution of force should prevail and consequently more simultaneous failure of the tissue components. Indeed, we have observed more abrupt failure in fresh tissue in which proteoglycan content was higher compared to matrices (p= 0.001) (Fig 3a). The failure phase was more gradual in matrices and not significantly different in duration (p= 0.646).

An important limitation of this study is that the mechanical test used did not allow determination of the mode of failure of the tissue as in the case of uniaxial tests [33], where the beginning of the failure is attributed to the commencement of breakage of collagen fibers. Nevertheless, since it is well acknowledged that uni- and biaxial tests do not adequately address the properties of anisotropic material [32, 20, 16, 19] and since this study focused on the properties of the leaflet as a whole, the burst test was preferred. While not providing a complete insight into the mode of failure it provided a measure of the bursting strength of the whole tissue at its weakest point.

It should be noted that while the majority of homografts currently implanted are cryopreserved, alternative methods of allograft preservation include storage in nutrient medium at 4°C which is also currently employed as well as earlier methods such as vitrification and freeze drying. Cryopreserved homografts show excellent long term clinical results [29]. Moreover, in addition to demonstrating that cryopreservation did not affect the collagen and proteoglycan extracellular matrix of allografts, Aidulis et al recently demonstrated that vitrification using 2,3-butanediol with or without polyethylene glycol followed by freezing in liquid nitrogen was impractical.
due to devitrification upon thawing, while freeze drying resulted in significant mechanical damage to the allograft tissue [2]. In this study we investigated cryopreservation in light of the reported excellent clinical performance of cryopreserved allografts. Nevertheless, satisfactory clinical results have also been reported with homografts stored in antibiotic supplemented medium despite poorer preservation of endothelial cells compared to cryopreservation [27, 24, 23] and suggests storage in antibiotic supplemented medium to be a viable alternative mode of preservation warranting investigation. Investigation of the use of this technique for aortic valve matrices however, was beyond the scope of the present study.

In conclusion: To our knowledge this is the first study to investigate the effects of cryopreservation on aortic valve matrices. Neither collagen nor proteoglycan content of porcine aortic valve matrices were significantly affected by cryopreservation. Cryopreservation, however, significantly affected the structural properties of porcine aortic valve matrices. While the ultimate strength of the tissue was not significantly affected, cryopreservation resulted in structural changes in the collagenous matrix and significantly increased the strain in matrices when pressurised. It is known that cryopreservation results in an alteration in the appearance of the histoarchitecture of heart valve tissue and it has been suggested that damage caused by cryopreservation is most likely due to ice formation within highly hydrated proteoglycan- and glycosaminoglycan-containing extracellular matrix [35]. Theoretically, in tissue valve engineering, matrices are only expected to be functional for a limited time until regenerated in vivo [45]. Determination of whether or not the increased strain observed in cryopreserved matrices significantly affects matrix durability warrants further investigations including tissue fatigue studies and in vivo testing.
Appendix 1

Conversion of load/extension curves to stress/strain curves

Stress/strain curves were calculated as follows. Appendix 1 Fig. 1, illustrates the dimensions used in the calculations.

**Using the XY-axes to base on the calculations**

1. The equation of the circle is calculated with as coordinates of the midpoint: \((3.5, h-r)\)
2. Then the equation of the tangent at the circle trough the origin \(O\) is calculated, and the slope \(m\) of this tangent is then found.
3. Then the coordinates of the point of contact \((X_a, Y_a)\) are calculated.
4. Based on these data, the mantle-area of the truncated cone \(OABD\) can be calculated: \[= \frac{1}{2} AO \times (\text{periphery of the circle } AB + \text{periphery of the circle } OD)\]
5. Also the area of the upper part \(ACB\) of the sphere can be calculated \(2\pi rh_2\).
6. The sum of these 2 areas (truncated cone + the part \(ACB\) of the sphere) = the area of the (expanded) valve during the test.
7. Based on the calculated area of the valve, the stress and % strain can be calculated at each point of a load/deformation curve: stress = load \((N)\) / area valve expressed as kPa; and % strain = (area valve – area opening in the plates) / area opening in the plates x 100.
**Formulae used in the calculations**

1. Slope of the tangent at the circle through the origin O:
   
   \[
   m = \frac{28(2.25 - h) + \sqrt{784(h - 2.25)^2 + 115(18h - 4h^2)}}{-57.5}
   \]

2. Coordinates of the point of contact (point A):
   
   \[
   X_a = \frac{mh - rm + 3.5}{m^2 + 1} \quad \text{and} \quad Y_a = mW_a
   \]

3. Mantle-area of the truncated cone OABD:
   
   \[
   C = \frac{\sqrt{X_a^2 + Y_a^2}}{2} [2\pi(3.5 - X_a) + 7\pi]
   \]

4. The area of the upper part ACB of the sphere:
   
   \[
   S = 2\pi r(h - Y_a)
   \]

5. Stress (kPa) at a certain point in the curve:
   
   \[
   = \frac{\text{load (N)}}{(C + S)}
   \]

6. Strain (%) at a certain point of the curve:
   
   \[
   = \frac{(C + S) - 12.25\pi}{12.25\pi} \times 100
   \]

Calculations were based on a value of 4.5 mm diameter of the sphere, and a diameter of 7 mm of the whole in the plates. In the calculations, friction due to the polished ball is assumed to be negligible.

**Appendix 2**

Appendix 2 Fig. 1, illustrates the burst test as opposed to a uniaxial or biaxial test. Heart valve leaflets are known to be anisotropic with a multidirectional collagen architecture and especially so in the central portion of the leaflets as tested. Generally the burst strength (BS) is proportional to the tensile strength (Force (N)) and square root of strain ($\varepsilon_{\text{max}}$) to failure (BS $\propto F \sqrt{\varepsilon_{\text{max}}}$), with a correction factor being applied to correct for the effect of fibre properties such as length and thickness [12]. As the plunger presses downwards, the leaflet is subjected to a complex of forces including radial, lateral and shear forces. Fibers lying under the plunger will experience mainly axial forces while those lateral to the plunger will experience mainly lateral forces (Appendix 2 Fig. 2). In all areas adhesion between fibers (extra-collagen-
ous matrix) will impact on the mechanical behaviour and ultimate burst strength of leaflets by affecting fibre realignment.

*Appendix 2 Fig. 1. Illustration of the burst pressure as opposed to a uniaxial and a biaxial test set up.*

*Appendix 2 Fig. 2. Axial and lateral forces applied to the collagen fibers during mechanical testing.*

**Appendix 3**

The ability of the different layers of the aortic valve to slide relative to each other when pressurised is well known. Such mechanical behaviour increases the load bearing ability of the tissue by reducing the magnitude of peak tensions developed as layers slide relative to each other [14]. As force is applied (Appendix 3 Fig. 1), elongation ($El$) of fibers in the clamping area (area I) is dependent on $\alpha_1$ and $d$ (where $\alpha_1$ = bending angle, and $d$ is a function of sample thickness, geometrical parameters of the device and depth of the plunger) and can be calculated from the relationship $El = \alpha_1 \times$
$r$ (radius of the arc depicted by $\alpha_1$ and the fibre being considered). As such the uppermost fibers will experience maximum elongation and the lowest fibers little or no elongation. In the area of force application (area III), the converse is true when one considers the arc described by the bending angle $\alpha_2$. In this area the lowest fibers will experience more elongation than the uppermost fibers. In area II, the pattern of elongation lies in between that of areas I and III. The opposing patterns of elongation between fibers in the upper and lower layers of the leaflet will result in considerable shear forces being developed within the tissue by translational movement of the upper and lower layers.

Appendix 3 Fig 1. Illustration of the influence of the applied force on the elongation of fibers depending on the valve leaflet area.

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Heart valve leaflets are coated with a layer of endothelial cells which may be genetically different from endothelial cell from other sites, but the extent and nature of these differences are not known [1]. Indeed an interesting observation in this regard, is that in experimental heart transplantation with massive myocardial rejection, little inflammation is observed in the valves [2]. Earlier studies performed by Filip and co-workers [3] suggested that interstitial cells of heart valves possess morphological and functional characteristics similar to smooth muscle cells. These authors showed that valvular interstitial cells, like fibroblasts were devoid of basal lamina, have long thin cytoplasmic extensions and were intimately associated with the extra-cellular matrix. Characteristically, they possessed well developed rough endoplasmic reticulum and Golgi apparatus and are extremely rich in micro and intermediate filaments. Like smooth muscle cells they were coupled by communicating (gap) junctions, contained actin filaments and dense bodies, expressed cGMP-dependent protein kinase and were closely apposed to motor nerve terminals. Moreover, they were capable of contraction in vitro. More recently, Taylor and co-workers [4] demonstrated and confirmed that valvular interstitial cells comprised a heterogenous cell population whose major phenotype was that of myofibroblasts. Valvular interstitial cells are believed to continuously synthesize, remodel and replenish the connective tissue matrix [5].

The origins of valvular interstitial cells and their replenishment are not fully understood. Evidence [6,7] suggest that the principal source of cells in both the fetus and adults is the overlying endocardium, whereby cells undergo an epithelial to mesenchymal transformation and migrate into the matrix. Cells from two other extra-cardiac sources are also found in developing valves, namely epicardial cells and neural crest cells. There is also evidence suggesting that the epithelial-mesenchymal transformation continues post-natal [8].

Several authors have reported on the use of interstitial cell isolates to seed matrices in heart valve engineering [9-11]. These isolates were invariably obtained from vascular cells. To date, a functional tissue engineered valve has not been developed and the density of cells needed to achieve repopulation of a matrix, with current technology, is unknown. In preliminary evaluations of vascular interstitial cells we established that these cell populations were not made up purely of myofibroblasts. Hence we report in this chapter, a model to procure myofibroblasts in large quantities for cell seeding by transdifferentiating fibroblasts in interstitial cell isolates using Trans-
forming Growth Factor-β1. In addition to vascular interstitial cells we investigated the transdifferentiation of dermal mesenchymal cells since the procurement of these cells involves less invasive methods compared to vascular cells. Investigations to seed matrices in our laboratory, which are still underway, revealed that after transdifferentiation, myofibroblasts did not sufficiently proliferate within the matrices after apparent invasion. It has been shown that the biological responses of proliferation and transdifferentiation were mutually exclusive events in fibroblasts and that these responses to TGF-B1 are regulated via a CTGF-dependent pathway in concert with either EGF or IGF-2 [12]. Consequently, under TGF-B1 stimulation the cells will either proliferate or differentiate. We hypothesized that the myofibroblasts in our seeding experiments were transdifferentiating at the expense of proliferation. In the second part of this chapter we establish a cocktail of growth factors including TGF-β1, to overcome TGF-β1 imparted inhibition of proliferation of fibroblasts and to promote their invasion, namely bFGF, EGF and TGF-β1.

References


Chapter 5
Transforming Growth Factor-β-Induced Transition of Fibroblasts: A Model for Myofibroblast Procurement in Tissue Valve Engineering

Kishan Narine, Olivier DeWever, Koen Cathenis, Marc Mareel, Yves Van Belleghem, Guido Van Nooten
Transforming Growth Factor-β-Induced Transition of Fibroblasts: A Model for Myofibroblast Procurement in Tissue Valve Engineering

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Background and aim of the study: The selection of a suitable cell type for scaffold seeding, its isolation and adequate expansion in vitro remains a major challenge in tissue valve engineering. The study aim was to establish a model for efficient procurement of myofibroblasts for in-vitro seeding using fibroblasts as progenitor cells.

Methods: Dermal and arterial mesenchymal cells from human (hDMC1.1 and hAMC1.1) and sheep (sDMC1.1 and sAMC1.1) were isolated by sequential culture. Cell isolates were characterized by stringent criteria based on morphology, immunocytochemistry using antibodies to vimentin, cytokeratin, prolyl 4-hydroxyxlase, smooth muscle α-actin (α-SMA) and smooth muscle myosin, and by Western blotting for α-SMA and N-cadherin. Cultures with less than 10-20% α-SMA-positive cells were considered to be fibroblastic. Cultures were subsequently transdifferentiated with the cytokine transforming growth factor-β1 (TGF-β1) during five days, and then evaluated morphotypically, by immunocytochemistry, and by Western blotting. The metabolic and functional properties of TGF-β1-treated and untreated colonies were compared by measuring the expression of extracellular proteins (collagen type 1 and tenasin-C1) and by a collagen matrix contraction assay.

Results: TGF-β1 successfully transformed both human and sheep fibroblasts to metabolically active and functional myofibroblasts based on stringent criteria for myofibroblast characterization. α-SMA positivity of 100% was obtained in all cases (hDMC1.1, hAMC1.1, sDMC1.1, and sAMC1.1) after transformation compared to less than 50% in the non-transformed state (hAMC1.1, 17%; hDMC1.1, 10%; sAMC1.1, 43%; and sDMC1.1, 30%). This observation was further supported by increased contractility and an up-regulation of extracellular protein production in transdifferentiated cells.

Conclusion: Untreated arterial cell isolates were, at best, less than 50% α-SMA-positive. By allowing procurement of high densities of myofibroblasts in a relatively short time, the model was seen to be a potentially useful tool in tissue valve engineering, at least in investigations using autologous cells in the sheep model.

The Journal of Heart Valve Disease 2004;13:281-289

Intense research into the tissue engineering of heart valves has resulted from the limitations of currently available heart valve prostheses, including both mechanical and bioprosthetic heart valves (1). Whilst mechanical valves are thrombogenic and require long-term anticoagulation therapy with its associated risks (2-5), bioprosthetic devices (derived from xenogenic and human tissue) are more biocompatible but less durable. Once manufactured, bioprosthetic valves are no longer biologically active, and their cells are unable to replenish or remodel the extracellular matrix. Consequently, these valves are prone to deterioration (6,7). Moreover, all currently available prosthetic valves lack the ability to grow. A tissue-engineered valve developed using viable cells, with its potential to grow and maintain the extracellular matrix, offers the potential to overcome these limitations. However, many challenges remain in tissue valve engineering, including the selection of a suitable cell type, its isolation, separation and adequate expansion in vitro for use in seeding biological and synthetic scaffolds.

The histological structure of human and porcine cardiac valve leaflets has been well documented (8-11). Each valve leaflet is composed predominantly of structural (collagen and elastin) and cellular (interstitial and
endothelial cells) elements that organize to form three distinct layers: (i) the ventricularis; (ii) the spongiosa; and (iii) the fibrosa. The cellular and structural elements are surrounded by proteoglycans, which are most predominant in the spongiosa layer. Recent interest in the tissue engineering of cardiac valves has led to more thorough investigations of valve leaflet mesenchymal cells. The latter has been reported as comprising a heterogeneous phenotypic and functional fibroblastic cell population. Moreover, it has been suggested that myofibroblasts represent the major phenotype of valvular interstitial cells (9,12). In addition to contractile properties, cardiac valve interstitial cells have also been shown to possess secretory properties (8,11).

The study aim was to characterize arterial and dermal mesenchymal cells from human and sheep using stringent morphological and immunocytochemical criteria. The feasibility of obtaining higher densities of myofibroblasts for cell seeding in tissue valve engineering by treating fibroblasts from these sources with the cytokine transforming growth factor-β1 (TGF-β1) was investigated. The seeding of higher densities of myofibroblasts could yield more densely populated matrices which are more representative of the native aortic valve.

Materials and methods

Cell culture

Arterial and dermal mesenchymal cell lines were established by using fresh tissue fragments from human pulmonary and sheep carotid arteries, human neonatal foreskin and sheep subcutaneous tissue. Fresh tissue fragments were maintained for 30 min in Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma, St. Louis, MI, USA) supplemented with 10-fold the normal concentration of antibiotics (fungizone 25 μg/ml, penicillin 100 μg/ml, streptomycin 1 mg/ml). The core was then cut into discs and placed in a 6-well tissue plate. An aliquot (100 μl) of pure fetal bovine serum (FBS; Sigma) was placed on top of the explants to promote adhesion to the substrate. After 24 h, a 3-ml aliquot of DMEM supplemented with 10% FBS and antimycotics and antibiotics (fungizone 2.5 μg/ml, penicillin 100 μg/ml, streptomycin 100 μg/ml) was added to each well. The explants were incubated at 37°C in 10% CO2 with a change of medium every 48 h, or as necessary. Mesenchymal cells migrated out of the explants and attached to the tissue culture dish. When the cells had reached confluence, the explants were removed and the cells passaged. Confluent flasks split at 1 in 3 became confluent within 7 days. Cells were passaged using 0.25% trypsin in 1 mM EDTA and used

Figure 1: Schematic representation of the collagen contraction assay. (Reproduced with permission from Grinnell et al., The Journal of Cell Biology, 1994;124:401; © Rockefeller University Press.)

Figure 2: Confluent cultures on plastic substrate. Human arterial (hAMC.1.1), human dermal (hDMC.1.1), sheep arterial (sAMC.1.1) and sheep dermal mesenchymal cells (sDMC.1.1) show spindle-shaped cells with slender processes.
for experiments from passages 1 to 3. The following primary cultures were obtained: human arterial mesenchymal cells (hAMC1.1 to 1.7), sheep arterial mesenchymal cells (sAMC1.1 to 1.4), human dermal mesenchymal cells (hDMC1.1 and 2.1) and sheep dermal mesenchymal cells (sDMC1.1 to 1.6). (The first number in the acronym indicates the source and the second number is the identification of the primary culture.)

Antibodies and reagents

The following primary antibodies were used: anti-smooth muscle α-actin (α-SMA) (clone 1A4), anti-fasenin-C (TNC) (clone BC-24), anti-pancytokeratin (clone C-11), anti-vimentin, anti-collagen type I (clone col I) and anti-tubulin (clone B-5-1-2) (all from Sigma), and anti-human prolyl 4-hydroxylase (DAKO, Glostrup, Denmark). Secondary antibodies coupled to horseradish peroxidase and antibodies coupled to fluorescein or biotin were obtained from Amersham Pharmacia (Buckinghamshire, UK). Recombinant TGF-β1 was from R&D systems (Minneapolis, MN, USA), and collagen type I from Upstate Biotechnology (Lake Placid, NY, USA).

Protein analysis

Cultured mesenchymal cells (0.5-1.0 × 10^5 cells per coverslip) were plated onto sterile coverslips, placed in 24-well plates containing DMEM supplemented with 10% FBS, and left for one day. Cells were then washed with serum-free DMEM and treated for one week with serum-free DMEM supplemented with TGF-β1 (1 ng/ml) or vehicle alone. Cells were then fixed and permeabilized by immersing the coverslips in ice-cold methanol for 10 min. Coverslips were washed in Tris-buffered saline (TBS) and blocked with TBS + 5% bovine serum albumin (BSA) for 30 min at room temperature. Primary antibodies, recognizing α-SMA (1/500), vimentin (1/50), cytokeratin (1/50), prolyl 4-hydroxylase (1/100) and TNC (1/100), were diluted in TBS and applied to coverslips for 1 h at room temperature. Coverslips were rewarshed in TBS and consequently incubated with secondary antibodies and 4',6-diamino-2-phenylindol (DAPI) for 1 h at room temperature. Coverslips were washed in TBS, dehy-

Figure 3: A, B) Immunocytochemistry of isolated cells. Human arterial and dermal mesenchymal cells (hAMC1.1 and hDMC1.1) are 100% positive for prolyl 4-hydroxylase and vimentin, but negative for cytokeratin and smooth muscle myosin.

Figure 4: A, B) Effect of TGF-β1 on smooth muscle α-actin (α-SMA). TGF-β1 treatment resulted in the up-regulation of α-SMA in human arterial (hAMC1.1), human dermal (hDMC1.1), sheep arterial (sAMC1.1) and sheep dermal (sDMC1.1) mesenchymal cells compared to untreated colonies. In all cases, TGF-β1 treatment resulted in an up-regulation of α-SMA.
drated and mounted on glass microscope slides. Samples were viewed using fluorescence microscopy (Dialux 20; Leitz, Wetzlar, Germany) and photographed using a Leitz Orthomat E camera system. The number of positively stained cells for each antigen was assessed quantitatively and expressed as a percentage. No significant staining was observed if coverslips were incubated with secondary antibody only.

**Western blotting**

Western blotting was performed as previously described (13). Briefly, cells were lysed with Laemmli buffer, after which equal amounts of protein were boiled and separated using SDS-PAGE. Proteins were transferred onto Hybond membrane (Amersham). To perform dot-blot analyses, cells were lysed with Laemmli buffer, and equal amounts of native protein were spotted onto Hybond membrane and dried. For immunostaining, blots were quenched with 3% BSA in phosphate-buffered saline (PBS) containing 0.5% Tween-20. The membranes were incubated with primary antibody, followed by three 5-min washes and incubation with horseradish peroxidase-conjugated secondary antibodies. Specific antibody binding was detected using an enhanced chemiluminescence system (Amersham). Scanning densitometry was performed by means of the Quantity One program (Bio-Rad; Hercules, CA, USA).

**Collagen contraction assay**

Mesenchymal cells cultured in 25 cm² plastic Falcon flasks were treated for one week with serum-free DMEM supplemented with TGF-β1 (1 ng/ml) or vehicle alone. Refreshments were performed on day 3 and day 5. Thereafter, cells were trypsinized and counted. Plates (24-well) were filled with 300 µl of neutralized type I collagen mixed with 2.5 x 10⁶ treated mesenchymal cells in suspension. Immediately after polymerization, 500 µl DMEM containing 10% BSA was added to each well, and the gels were mechanically loaded by incubation at 37°C during 24 h. To initiate contraction, the gels were detached with a spatula and gentle shaking of the culture wells until they floated in the medium (Fig. 1). Collagen gel cultures of both TGF-β1-pre-treated and non-treated cells were maintained in the culture medium without TGF-β1 during the experimental period. The diameters of the gels were measured at each time point to calculate the percentage contraction using the formula: [diameter time 0 - mean diameter time X]/diameter time 0, expressed as percentage, wherein X represents the number of hours after TGF-β1 treatment.

**Results**

**Morphology of cell isolates**

Confluent cultures of hDMC1.1, hAMC1.1, sDMC1.1 and sAMC1.1 after seven days are illustrated in Figure 2. Morphotypically, cultures from both sheep and human sources were composed of spindle-shaped cells with slender processes consistent with mesenchymal fibroblastic colonies. In all experiments, cells from low-passage culture (less than five) were used.

Human mesenchymal cells (hAMC1.1 and hDMC1.1) were further characterized by immunocytochemical staining for vimentin, prolyl 4-hydroxylase,

| Table I: Immunocytochemistry and Western blotting of isolated human arterial (hAMC1.1), human dermal (hDMC1.1), sheep arterial (sAMC1.1) and sheep dermal (sDMC1.1) mesenchymal cells. |
|---------------------------------|-------------------------------------------------|----------------------------|---------------------------------|---------------------|
| **Cell line**                  | **Marker**                                      | **Immunocytochemistry**    | **Western blot**                |
| **Tissue of Origin**            | **Acronym**                                     | Vimentin       | Cytokeratin       | Prolyl 4-hydroxylase | Smooth muscle myosin | α-SMA | N-cadherin | α-SMA |
| Human pulmonary artery          | hAMC1.1                                        | +             | -                | +                   | -                   | 17%+ | +         | +     |
| Sheep carotid artery            | sAMC1.1                                        | ND            | ND               | ND                  | ND                  | 43%+ | +         | +     |
| Human foreskin                  | hDMC1.1                                        | +             | -                | +                   | -                   | 10%+ | +         | +     |
| Sheep dermis                    | sDMC1.1                                        | ND            | ND               | ND                  | ND                  | 30%+ | +         | +     |

ND: Not detected; α-SMA: Smooth muscle α-actin.
smooth muscle myosin and cytokeratin. Human arterial mesenchymal cell cultures were negative for cytokeratin and smooth muscle myosin, but 100% positive for prolyl 4-hydroxylase and vimentin (Table I; Fig. 3). Sheep arterial and dermal mesenchymal cells were not stained for the above-mentioned markers, as antibodies that cross-react with these sheep proteins are not available. All isolated primary cell lines were positive for N-cadherin. After characterization, cultures were stored frozen, and thawed as required for further investigation.

Morphotype, protein synthesis and collagen contraction of transdifferentiated cells

Thawed cell lines were analyzed comparatively before and after treatment with TGF-β1 for five days.

The effect of TGF-β1 on α-SMA expression in hAMC1.1, hDMC1.1, sAMC1.1 and sDMC1.1 is shown in Figure 4. In all cases, TGF-β1 resulted in an up-regulation of α-SMA expression with pronounced stress fibers in mesenchymal cells. The number of cells in comparative colonies was the same, as evidenced by nuclei staining using DAPI. TGF-β1-treated cells were 100% positive for α-SMA compared to untreated cells (Table II).

These findings were confirmed by Western blotting, which showed an increase in α-SMA after TGF-β treatment in all cell lines (Fig. 5).

Expression of the extracellular proteins; collagen type I and TNC

Collagen type-I and TNC expression were both up-regulated in TGF-β1-treated cells.

| Cell type | α-SMA expression (%)
<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>hDMC1.1</td>
<td>10</td>
</tr>
<tr>
<td>hDMC1.1 + TGF-β1</td>
<td>85</td>
</tr>
<tr>
<td>hAMC1.1</td>
<td>17</td>
</tr>
<tr>
<td>hAMC1.1 + TGF-β1</td>
<td>100</td>
</tr>
<tr>
<td>sDMC1.1</td>
<td>30</td>
</tr>
<tr>
<td>sDMC1.1 + TGF-β1</td>
<td>100</td>
</tr>
<tr>
<td>sAMC1.1</td>
<td>43</td>
</tr>
<tr>
<td>sAMC1.1 + TGF-β1</td>
<td>100</td>
</tr>
</tbody>
</table>

Collagen production was monitored using two techniques: (i) indirectly, by investigating the enzyme prolyl 4-hydroxylase which is essential for the maturation of collagen type I; and (ii) directly, by performing a dot-blot assay on the total cell lysate. Dot-blot analysis showed increased collagen production in TGF-β1-treated human mesenchymal cells (Fig. 6). An irrelevant antibody IgG showed no significant staining, and tubulin staining confirmed a fixed protein concentration in analyzed blots. These results showed a concomitant increase in prolyl 4-hydroxylase staining, and suggested an increased collagen synthesis in hAMC1.1 and hDMC1.1 (Fig. 3B; Table II).

Immunocytochemical analysis of TNC in hAMC1.1 and hDMC1.1 is illustrated in Figure 7. In both cases, TGF-β1 resulted in an up-regulation of TNC.

Collagen contraction assay

The rate and extent of collagen contraction in both sheep and human mesenchymal cells treated with
TGF-β1 is shown in Figure 8. Both the rate and extent of collagen contraction was increased in the transdifferentiated cells. Indeed, transdifferentiated arterial cells contracted more rapidly and to a greater extent than did transdifferentiated dermal cells.

Discussion

The role of fibroblasts in the maintenance of heart valve extracellular matrix has been extensively reported (14-16). In addition to structural proteins, valve interstitial cells have been shown to express growth factors, cytokines and chemokines. More recently, Taylor and co-workers elegantly illustrated the expression of the fibroblastic cell markers prolyl 4-hydroxylase, β-tubulin and vimentin by some valve interstitial cells (9).

It has also been shown that, in addition to expressing markers similar to skeletal, cardiac and smooth muscle cells, many valve interstitial cells express α-SMA, which is characteristic of myofibroblasts. Fibroblastic cells expressing α-SMA were also shown to be associated with contractile properties and rapid remodeling of the extracellular matrix (17,18).

Several authors have reported on the use of interstitial cells to seed biological and synthetic matrices in tissue valve engineering (19-23). Particularly encouraging were the investigations of Shinoka, Breuer and co-workers, who used mesenchymal cells from sheep carotid arteries. These authors reported the tissue engineering of complete pulmonary leaflets from autologous sheep mesenchymal cells seeded onto biodegradable polyglycolic acid scaffolds and implanted into juvenile sheep for up to 24 weeks. In the above-mentioned reports, cells on tissue-engineered constructs were evaluated phenotypically and functionally after placement either in sheep or in a hemodynamic culture chamber. The demonstration of myofibroblasts (α-SMA) in the latter cell populations has generally been taken as evidence of the constructs’ potential to mimic the native aortic valve. Although native valves are predominantly composed of myofibroblasts, the same cannot be said for mesenchymal cell isolates, as discussed below.

The stimulatory effects of TGF-β on mesenchymal cells are known. The isoforms TGF-β1, TGF-β2 and TGF-β3 belong to the TGF-β superfamily of cell-cell signaling proteins, the pleiotropic effects of which are mediated from cell membrane to nucleus by type I and type II serine/threonine kinase receptors and their downstream effectors known as Smad proteins (24,25).

In the present study, the morphotypes of cells on a plastic substrate were in keeping with that of fibroblastic mesenchymal cells, and were considered as such in this study. Isolated cells expressed vimentin (the intermediary filament of mesenchymal cells) and prolyl 4-hydroxylase, an enzyme that synthesizes hydroxyproline in collagen synthesis (26).

At the same time, these cultures were negative for smooth muscle heavy chain polypeptide (200 kDa) of

![Figure 7: Immunocytochemistry of extracellular proteins. Tenascin-C was up-regulated in TGF-β1-treated human mesenchymal cells, as shown for human arterial mesenchymal cells (hAMC.1.1).](image)

![Figure 8: A, B) Collagen gel contraction. Both the rate and extent of collagen contraction was increased in TGF-β1-treated human (hAMC.1.1 and hDMC.1.1) and sheep (sAMC.1.1 and sDMC.1.1) mesenchymal cells.](image)
myosin and cytokeratin, the intermediary filament found primarily in epithelial cells. These findings indicate that isolated cells were of mesenchymal cell type and actively synthesizing collagen. Neither cells isolated from arterial nor dermal tissue were homogeneously positive for α-SMA, but comprised at least two phenotypes depending on whether or not they expressed α-SMA; this was in keeping with findings reported by others (9).

Smooth muscle α-actin is expressed by, and is characteristic of, myofibroblasts. The percentage of cells expressing α-SMA in isolated cultures ranged from 10 to 43% - a factor that might be limiting when these isolates are seeded onto scaffolds in tissue valve engineering. Interestingly, sAMC.1.1 and sDMC.1.1 express significantly more α-SMA compared to hAMC.1.1 and hDMC.1.1. Whilst such variation may be species-dependent, sAMC.1.1 were derived from canid arteries subjected to systemic circulatory pressures, while hAMC.1.1 were derived from pulmonary arteries in the lower-pressure pulmonary circulation. The transdifferentiation of fibroblasts to myofibroblasts in response to tension has been documented. Arora and co-workers (18) demonstrated an association between local intracellular tension and the expression of α-SMA by fibroblasts. This observation could, at least partially, explain the difference in α-SMA expression in untreated hAMC.1.1 and sAMC.1.1.

TGF-β1-treated cells were 100% positive for α-SMA, as shown by immunocytochemistry and Western blotting, and this indicated a transition to myofibroblasts that exhibited pronounced longitudinal stress fibers. The use of such transdifferentiated cell lines from both sheep and human in tissue valve engineering would result in higher densities of myofibroblasts in seeded cells. The ability of fibroblasts to transdifferentiate to a myofibroblastic phenotype with successive passages has been reported (27). This transdifferentiation implies a relationship between the number of cell passages and α-SMA expression. Whilst this phenomenon was not investigated in the present studies, it should be noted that early-passage cells were used in these studies.

TGF-β1-treatment resulted in an up-regulation of the extracellular proteins collagen and TNC. Up-regulation of collagen synthesis was evidenced by an increase in total collagen and an accompanying increase in prolyl 4-hydroxylase activity. TNC is a disulfide-linked hexameric matricellular glycoprotein that is prominent in embryonic and adult tissues that are actively remodeling, such as in wound healing and cancer, where TNC is often expressed by myofibroblasts (28). When present in mixed substrata, the matricellular TNC protein may antagonize the pro-adhesive activities of other matrix proteins, such as collagen type I (29). TNC can modulate cell mobility, and might therefore be important for the invasion of myofibroblasts into a three-dimensional collagen matrix.

Caderhins are transmembrane glycoproteins linked to the actin cytoskeleton via the catenins, α-catenin and β-catenin or γ-catenin; N-cadherin-cadherin is a path-finding, promigratory molecule which is expressed in fibroblasts, myofibroblasts, neurons, smooth muscle cells and endothelial cells. The presence of N-cadherin at cell-cell borders of myofibroblasts may provide a site for the insertion of α-SMA filaments. An N-cadherin-actin association could support actin-based force generation in valve leaflets and efficient migration.

It has been reported that TGF-β-treated fibroblasts (myofibroblasts) contract a collagen gel more rapidly and to a greater extent than do fibroblasts (30). Consequently, in the present studies fibroblastic cultures were pretreated with 1 ng/ml TGF-β1. Mechanically loaded (stressed) collagen gel contractions were performed as described, and contraction was easily measurable after a few hours. These results indicated that TGF-β1-pretreated cells contract collagen gel faster and to a greater extent than do untreated cells - an observation which also suggests that the myofibroblastic phenotype is maintained in the absence of TGF-β1.

Arterial mesenchymal cells contracted collagen more than dermal cells under both control and TGF-β1-treated conditions, and are consistent with higher α-SMA expression, as shown by immunocytochemistry in arterial cells.

In conclusion, early-passage human and sheep arterial and dermal mesenchymal cells contained less than 50% myofibroblasts. Arterial mesenchymal cells isolated however, contained more myofibroblasts than dermal isolates in both sheep and human cells. TGF-β1 successfully transdifferentiates arterial and dermal fibroblasts from human and sheep to metabolically active and functional myofibroblasts. Nevertheless, the use of dermal mesenchymal cells is a less-invasive alternative to arterial mesenchymal cells as a source of myofibroblasts using this model. Moreover, the model is potentially useful in obtaining high densities of myofibroblasts for use in tissue valve engineering. The ability of these cells to repopulate biological matrices is currently being investigated by the present authors.

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Meeting discussion

DR. PATRICIA TAYLOR (United Kingdom): For how long do you expect this effect to last?

DR. KISHAN NARINE (Ghent, Belgium): When the TGF-β treatment is removed, and the cells are transdifferentiated, the effect ceases - that seems to be the case.

DR. TAYLOR: Have you looked at the cells later on to see if they have maintained the phenotype?

DR. NARINE: Yes - when the cells were removed from TGF-β treatment, the contractility increased for a few hours. So the phenotype was met even in the absence of TGF-β for a short interval. There is a trend that once the cells are transdifferentiated they will stay that way.

DR. ROBERT GALLEGOS (USA): You speak of a desire to obtain larger sources of mesenchymal cells. Have you tried using bone marrow-derived mesenchymal stem cell lineages? Could this system be used to test whether it is the environment that may induce differentiational expression?

DR. NARINE: Yes, we have, and this forms part of our ongoing experiments. We are actually looking at circulating stem cells.

DR. GALLEGOS: Do you see any difference in terms of expression of, for example, the collagen content of these cells rather than cells derived from venous or arterial sites?

DR. NARINE: No.

DR. PETER ZILLA (South Africa): TGF-β is probably the most colorful growth factor, with various facets and contradictory effects regarding collagen storage. Might you be playing with fire by tissue engineering valve leaflets where you need to retain a very thin dimension but end up with a fibrotic leaflet?

DR. NARINE: Perhaps. But in many previous reports where isolated cells have been re-sewn onto valves and the valves then either implanted into animals or used in bioreactors, the cells have transdifferentiated to myofibroblastic phenotypes. This was a way of achieving a head’s start by sewing myofiblasic phenotypes onto the matrices. But this is not a cancerous situation, if that is what you are inferring.

SIR MAGDI YACOUB (United Kingdom): Have you looked at the specific receptor subtype through which that effect in this cell is being mediated? In other words, have you tried to block it with specific receptor blockers, because TGF-β is a molecule with many, many facets and produces opposite effects through specific receptors. It really is the receptor which is more important.

DR. NARINE: No, we have not. I agree that there is some fear about TGF-β, especially the association with cancerous cells. We know that myofibroblasts are at the forefront of cancer invasion, and that TGF-β does play a role here. However, it is not the effect of TGF-β on the cells that is an important factor in the invasion process and the production of an extracellular matrix for the invasion of cancer cells - it may be simply a recruiting factor rather than a transdifferentiating factor in a cancer setting. But we must wait for further results.
Chapter 6
Growth Factor Modulation of Fibroblast Proliferation, Differentiation, and Invasion: Implications for Tissue Valve Engineering

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Growth Factor Modulation of Fibroblast Proliferation, Differentiation, and Invasion: Implications for Tissue Valve Engineering

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ABSTRACT

We have previously shown that transforming growth factor-beta1 (TGF-β1) stimulates trans-differentiation of fibroblasts into smooth muscle α-actin (α-SMA) positive myofibroblasts. However, TGF-β, as such, is unsuitable for effective population of a heart valve matrix, because it dose-dependently inhibits growth of fibroblasts. The aim of this study was to investigate combinations of other growth factors with TGF-β to stimulate the proliferation of suitably differentiated cells and to enhance their invasion into aortic valve matrices. Human dermal mesenchymal cells (hDMEC1.1) were treated with combinations of growth factors to stimulate these cells to trans-differentiate into myofibroblasts, to proliferate, and to invade. Growth factors were chosen after expression of their respective receptors was confirmed in hDMEC1.1 using reverse transcriptase polymerase chain reaction. We combined TGF-β with several growth factors such as insulin-like growth factor (IGF-1, IGF-2), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF-AA, PDGF-BB, and PDGF-AB). Nuclear Ki67 staining, (MTT) assay and cell counting revealed that only EGF and bFGF were capable of overcoming TGF-β-induced growth inhibition. However, bFGF but not EGF inhibited TGF-β-induced α-SMA expression, as evidenced by immuno-cytochemistry and Western blotting. A growth factor cocktail (TGF-β, EGF, bFGF) has been established that maintains TGF-β-induced trans-differentiation but overcomes TGF-β-induced growth inhibition while stimulating fibroblast proliferation and invasion.

INTRODUCTION

Construction of a replacement tissue-engineered aortic heart valve poses several challenges.1 Nevertheless, its potential advantages over contemporary prostheses have prompted intense research aimed at developing a living construct that mimics the native aortic valve biologically and functionally.2,3 A vital tissue construct capable of maintaining its extracellular matrix would avoid the risks of anticoagulation therapy and early deterioration, which are associated with current mechanical and biological prostheses, respectively.4,5 The cellular component of aortic valve leaflets consists of interstitial cells embedded within the leaflet, which in turn is lined by a layer of immune-competent endothelial cells. It has been reported that valvular interstitial cells comprise a heterogeneous phenotypic and functional fibroblastic cell population, with myofibroblasts representing the major phenotype.6

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One approach to tissue valve engineering involves the in vitro re-population of acellular biological matrices with interstitial cells and requires invasion of matrices by sufficient functional cells. Several polypeptide growth factors have been implicated as extracellular signal molecules in the intracellular regulation of cell proliferation, trans-differentiation, and invasion via enzyme-linked transmembrane receptors.

The study aim was to establish a growth factor combination that could enhance the proliferation, differentiation, and invasion of fibroblasts and thus potentially useful in tissue valve engineering. The polypeptide growth factors basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), platelet-derived growth factors (PDGF) -AA, -AB, and -BB, insulin-like growth factor (IGF)-1 and -2, and transforming growth factor beta 1 (TGF-β1) were evaluated separately and in combination using human dermal mesenchymal cells (hDMCs). Human dermal fibroblasts were used in this study because they can be harvested with minimal invasion from potential valve recipients and are as such a more-attractive source of progenitor cells for myofibroblasts than other potential sources such as arterial fibroblasts.

**MATERIALS AND METHODS**

hDMC1.1 were characterized morphologically and immunocytochemically. Expression of receptors for the polypeptide factors bFGF, EGF, IGF-I, IGF-2, PDGF-AA, PDGF-AB, PDGF-BB, and TGF-β1 in hDMCs were determined using reverse transcriptase polymerase chain reaction (RT-PCR) and Western blot analysis. The effects on the differentiation, proliferation, and invasion of hDMCs were investigated after establishing the expression of their corresponding receptors in hDMCs. Growth factor concentrations of 10 ng/ml, were used based upon corresponding dose–response studies of their effects on proliferation and smooth muscle α-actin (α-SMA) expression in primary dermal fibroblasts (data not shown).

**Cell culture**

Mesenchymal cell lines from human neonatal foreskin were established as previously described.

**Antibodies, polypeptide growth factors, PCR primers, and reagents**

The following primary antibodies were used: anti-α-SMA (clone 1A4), anti-tenascin-C (anti-TNC) (clone BC-24), anti Ki67, anti-N-cadherin, anti-tubulin (clone B-5-1-2), anti-EGF receptor (anti-EGFR) (all from Sigma, St. Louis, MO), anti-human prolyl-4-hydroxylase (PH4) (DAKO, Glostrup, Denmark), anti-FGF receptor (anti-FGFR-1), and anti-TGF-β receptor-2 (anti-TGF-βR-2), both from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies coupled to horseradish peroxidase and antibodies coupled to fluorescein were obtained from Amersham Biosciences (Uppsala, Sweden). Recombinant TGF-β1, PDGF-AA, PDGF-AB, PDGF-BB, IGF-1, IGF-2, EGF, and bFGF were obtained from Pepro Tech EC (London, UK).

Forward and reverse primers for the PCR amplification of the receptors EGF-R, FGFR-1, FGFR-2, TGF-βR-2, IGF-1R, IGF-2R, PDGF-β, and β₂-microglobulin (β₂-MG) were obtained from Invitrogen (Merelbeke, Belgium) and are shown in Table 1.

**Immunocytochemistry**

Cultured hDMC1.1 (0.5–1.0×10⁵ cells per coverslip) were plated onto sterile coverslips, placed in 24-well plates containing Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and incubated for 1 week. Cells were then fixed and permeabilized by immersing the coverslips in ice-cold methanol for 10 min. Coverslips were washed in Tris-buffered saline.

**Table 1. Forward and Reverse Primers for the Polymerase Chain Reaction (PCR) Amplification of the Receptors Epidermal Growth Factor Receptor (EGFR), Fibroblast Growth Factor Receptor (FGFR)-1, -2, Transforming Growth Factor-Beta 2 (TGF-βR-2), Insulin-Like Growth Factor (IGF)-1 Receptor (IGF1R), IGF-2R, Platelet Derived Growth Factor Receptor (PDGF)-α, PDGF-β, and β₂ Microglobulin (β₂-MG)**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>AAAGCCGCAAGGATTCGG</td>
<td>ATGGGGCAGGGGATACG</td>
</tr>
<tr>
<td>FGFR1</td>
<td>ATAGCTTGCGGAAAACACGAC</td>
<td>CCAAGTGCTGATGAGTGGTT</td>
</tr>
<tr>
<td>FGFR2</td>
<td>ATGAGCGGAGGAAAACACGAC</td>
<td>TCTCGAGCAGCAGTGTG</td>
</tr>
<tr>
<td>TGF-βR-2</td>
<td>GGCGGGGCGGAAAAACACGAC</td>
<td>TCTCGAGCAGCAGTGTG</td>
</tr>
<tr>
<td>IGF1R</td>
<td>TGCCCAACCCCTTACTGGA</td>
<td>CAGCAGTGCTGCTGAAATG</td>
</tr>
<tr>
<td>IGF2R</td>
<td>GTGCGCATTCTATGCGG</td>
<td>TCTCGAGCAGCAGTGTG</td>
</tr>
<tr>
<td>PDGF-α</td>
<td>AGTCGAGGGAAGGATGGTGG</td>
<td>TCTCGAGCAGCAGTGTG</td>
</tr>
<tr>
<td>PDGF-β</td>
<td>CTCACATGAGGATCCTGAG</td>
<td>TCTCGAGCAGCAGTGTG</td>
</tr>
<tr>
<td>β₂-MG</td>
<td>CATCCAGCCTTAGTCCAAAGA</td>
<td>GACAGTGTGCTTAAAG</td>
</tr>
</tbody>
</table>

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(TBS) and blocked with TBS + 5% bovine serum albumin (BSA) for 30 min at room temperature. Primary antibodies, recognizing α-SMA (1/500), PHA (1/100), and Ki67, were diluted in TBS and applied to coverslips for 1 h at room temperature. Coverslips were washed in TBS and incubated with secondary antibodies and 4', 6-diamino-2-phenylindol (DAPI) for 1 h at room temperature. Coverslips were washed in TBS, dehydrated, and mounted on glass slides. The samples were viewed using the Axiosvert 200 microscope (Carl Zeiss, Göttingen, Germany).

**Western blot**

Western blot was performed as previously described. Briefly, cells were lysed with Laemmli buffer, after which equal amounts of protein were boiled and separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins were transferred onto Hybond membrane (Amersham). For immunostaining, blots were quenched with 3% BSA in phosphate-buffered saline (PBS) containing 0.5% Tween-20. The membranes were incubated with primary antibody, followed by three 5-min washes and incubation with horseradish peroxidase-conjugated secondary antibodies. Specific antibody binding was detected using an enhanced chemiluminescence system (Amersham).

**MTT assay** ([4,5-dimethyl-thiazol-2-yl]-2, 5-diphenyltetrazolium bromide assay)

MTT assay was performed by adding 20 μL of MTT ([4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution (Sigma-Aldrich, Bornem, Belgium) to each well at 37°C in a 10% carbon dioxide incubator for 2 h. After removal of media, crystals were dissolved in 100 μL dimethyl sulfoxide (Sigma Aldrich, Bornem, Belgium) and the absorbance measured at 490 nm. The optical density of untreated hDMC1.1 used as a control was assigned a value of 100 for comparative purposes. Consequently, a score above 100 indicated growth stimulation, and a score below 100 indicated growth inhibition.

Results were statistically analyzed using an unpaired, 2-sided t-test whereby each treatment was tested against the control.

The findings of the MTT assay were checked using Ki67 immune staining and DAPI. Cells were seeded onto coverslips (5.0×10^4 cells per coverslip) and incubated for 1 week in 500 μL DMEM supplemented with 2.5% FBS and the corresponding growth factor. Cells were stained with anti-Ki67 and DAPI.

**Expression of polypeptide receptors in hDMC1.1**

Receptor expression was determined by extraction of total ribonucleic acid (RNA) from hDMC1.1 and subsequent amplification using RT-PCR and the specific receptor primers for FGFR-1, FGFR-2, EGFR, TGF-βR-2, IGF-1R, IGF-2R, PDGF-α, and PDGF-β. Each primer set was selected using the Gene Runner 3.00 amplification program for products of 400 bp, 500 bp, and 550 bp. To check for contamination of isolated RNA by remaining deoxyribonucleic acid (DNA), PCR was performed without RT. A primer pair for β2MG was used as a positive control. Consequently to their effects on proliferation and differentiation as described below, receptors for FGF, EGF, and TGF-β were confirmed in hDMC1.1 using Western blot analysis.

**RNA isolation and quantitative determination**

RNA was isolated from mesenchymal cells using the Rneasy Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer’s protocol. Cells were lysed in guanidium isothiocyanate extraction buffer supplemented with β-mercapto ethanol (Bio-Rad, Hercules, CA). The lysate was suspended in 70% Rnase-free ethanol (Merck, Darmstadt, Germany), transferred to an Rneasy mini column (Qiagen, Venlo, Netherlands) and centrifuged using Rnase-free filter tips (Sorenson Bioscience Inc., Salt Lake City, UT). Columns were each washed once with 700 μL (RPE) per column and then twice with 500 μL RPE. RNA was eluted once each using 20 μL and 15 μL Rnase-free water. Extracts were stored at −20°C for further use. RNA in solution (dilution 1/100) was quantified spectrophotometrically by measuring the absorbance at 280 nm.

**RT and PCR amplification**

The expression of polypeptide growth factor receptors in hDMC1.1 was investigated by RT PCR using the Qiagen Omniscript RT Kit and Qiagen Taq Polymerase Kit (Qiagen, Venlo, Netherlands). Isolated RNA template (1 μg) was added to a master mix containing Omniscript RT, buffer RT, deoxyribonucleotide triphosphate mix, RNAse inhibitor, and oligo-dT primers in RNase-free water. The concentration of complimentary DNA (cDNA) produced was determined using a DNA analyzer and subsequently amplified using PCR. Template cDNA was added to a master mix containing Taq DNA polymerase, Qiagen PCR buffer, magnesium chloride, deoxyribonucleotide triphosphate, forward primer, and reverse primer in RNase-free water. Mixtures were denatured at 95°C before the primers were annealed at 58°C and elongated at 72°C. Amplification products were visualized on agarose gel using ultraviolet illumination after intercalation of ethidium bromide.

**Effects of polypeptide factors on hDMC1.1 differentiation**

Cells (2.0×10^5 per well) were treated with each polypeptide factor for 1 week in growth medium supplemented with 2.5% FBS. The trans-differentiation of fibroblasts to myofibroblasts was analyzed using sodium dodecyl sulfate
polyacrylamide gel electrophoresis and Western blot using antibody to α-SMA stress fibers, which is characteristic of myofibroblasts and is upregulated in trans-differentiated fibroblasts.

Protein loading was checked by staining for tubulin with anti-tubulin antibody. Untreated hDMC1.1 were used as a control.

Subsequently, the effects of TGF-β, EGF, bFGF, and their combinations were investigated on individual fibroblasts by immunocytochemistry using anti-α-SMA. Furthermore, to check for a possible influence of the proliferative effects of these molecules on differentiation, treated cells were doubly stained with polyclonal Ki67 and monoclonal α-SMA.

**Effect of polypeptide factors on hDMC1.1 proliferation**

The effect of the polypeptide factors on the proliferation of hDMC1.1 was evaluated using MTT assay. hDMC1.1 (10.0×10^5 cells per well) were treated with polypeptide factors at a concentration of 10 ng/mL and incubated at 37°C for 1 week in 200 µL DMEM supplemented with FBS.

To avoid interference resulting from FBS growth stimulation, the concentration of FBS in the medium was reduced from 10% to 2.5%.

The polypeptide bFGF was supplemented with heparin at a concentration of 10 ng/mL. After testing each factor individually, bFGF and EGF were retained for further investigation and tested individually with TGF-β1 and as a combination with and without TGF-β1. The remaining factors were eliminated from the study.

The findings of the MTT assay were checked using nuclear antigen Ki67 immunostaining and DAPI. Cells were seeded onto cover slips (5.0×10^5 cells per coverslip) and incubated for 1 week in 500 µL DMEM supplemented with 2.5% FBS and polypeptide factor. Cells were fixed and stained with a polyclonal anti-Ki67 antibody and DAPI and viewed using fluorescence microscopy. The proliferation index was calculated using the formula: proliferation index = (number of Ki67-positive nuclei/total number of nuclei)×100%.

**Effect of polypeptide factors on expression of pro-invasive markers in hDMC1.1**

The effects of EGF, bFGF, TGF-β, and their combined treatment on hDMC1.1 were investigated by determining the expression of the pro-invasive markers, N-cadherin and TNC. We have previously shown and reported that both of these markers are pro-invasive.19,21 Cells were treated for 1 week with the corresponding polypeptides and analyzed using Western blot using anti-N-cadherin.

TNC was determined immunohistochemically by staining individual cells with monoclonal anti-TNC (BC-24). To check for a possible relationship between the proliferative effects of these molecules and invasion, double immunofluorescent staining using anti-TNC and anti-Ki67 was performed.

**RESULTS**

Morphological and Immunocytochemical characteristics of hDMC1.1

Mesenchymal cells had a spindle shaped morphology consistent with fibroblasts, as we have previously reported.19 Immunocytochemically (Fig.1), cultures were 100% positive for PH4 and showed a basal expression of

![FIG. 1. Immunofluorescent staining of human dermal mesenchymal cells using anti-prolyl-4-hydroxylase (PH4), anti-smooth muscle α-actin (α-SMA) and anti-Ki67 antibodies combined with 4', 6-diamino-2-phenylindol staining of the nucleus (insert). Scale = 50 µm.](image-url)
α-SMA. Ki67 staining was slightly positive, indicating basal cell proliferation. The insets show total cell counts using DAPI.

Expression of polypeptide receptors

Fig. 2 shows the electrophoretic pattern for the RT-PCR amplification product of RNA isolated from hDMC1.1 untreated hDMC1.1. β2 MG resulted in a band corresponding approximately to 165 base pairs and confirmed the procedure. No band was observed in the negative control. The polypeptide receptors FGFR-1, FGFR-2, EGFR, TGF-βR-2, IGF-1R, IGF-2R, PDGFR-α, and PDGFR-β were expressed in hDMC1.1. In the amplification of FGFR, EGFR, and PDGFR-β, additional bands were observed. Amplification of FGFR-2 resulted in several bands, whereas 1 extra band was observed for EGFR and a doublet for PDGFR-β. Western blot performed on hDMC1.1 cell lysate confirmed receptor expression (Fig. 2B).

Proliferation

Fig. 3A shows the relative optical density at 490 nm for hDMC1.1 treated with the polypeptide factors IGF-1, IGF-2, PDGF-AA, PDGF-AB, and PDGF-BB. Fig. 3B shows hDMC1.1 treated with TGF-β, bFGF, and EGF separately and in combination. There was no significant increase in proliferation after treatment with IGF-2 or TGF-β, whereas IGF-1, PDGF-AA, PDGF-AB, and PDGF-BB treatment resulted in significant but modest increases in proliferation.

An approximately twofold increase in optical density was observed after treatment with bFGF and EGF, indicating a corresponding increase in proliferation. This increase was approximately threefold when EGF and bFGF were applied in combination. TGF-β combined with EGF or bFGF resulted in significantly higher optical densities than TGF-β alone. The combination of bFGF and TGF-β gave a higher optical density than bFGF alone. On the other hand, the combination of EGF and TGF-β resulted in a lower optical density than EGF alone. The optical density of the triple combination, TGF-β, EGF, and bFGF, was significantly higher than the control although slightly lower than the approximately threefold increase seen with the combination of EGF and bFGF. Fig. 4 shows the antiKi67 staining and DAPI of treated cells. The proliferation indices are shown in Table 2. The pattern of proliferation confirmed the findings of the MTT assay.

Differentiation and upregulation

Fig. 5 shows the Western blot analysis for α-SMA expression in hDMC1.1 after treatment with TGFβ, bFGF, EGF, and their combinations. The control population showed only a low expression of α-SMA. TGFβ significantly upregulated expression of α-SMA. Neither EGF, bFGF, nor
FIG. 3. (A) Quantification of proliferation of human dermal mesenchymal cells. Cells were seeded in 200 µL Dulbecco’s modified Eagle medium supplemented with 2.5% fetal bovine serum and 10 ng/mL of the corresponding growth factor. The graph shows the relative optical density and standard deviation. (B) Relative optical density as in (A). The standard deviation of cells treated with a combination of transforming growth factor-beta (TGF-β) with other growth factor(s) was calculated relative to the TGF-β-treated group. Other standard deviations were calculated relative to the control, as in (A). IGF, insulin-like growth factor; PDGF, platelet derived growth factor receptor; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor.

FIG. 4. Ki67 expression in human dermal mesenchymal cells (hDMC1.1) after treatment with transforming growth factor-beta (TGF-β), fibroblast growth factor (FGF), basic FGF (bFGF), and their combinations using a polyclonal anti-Ki67 antibody. 4’, 6-diamino-2-phenylindol staining is shown in inserts. Scale bar = 50 µm. EGF, epidermal growth factor.

their combined treatment resulted in significant α-SMA upregulation. The combination of TGF-β and bFGF or EGF upregulated α-SMA. However, the combination TGF-β and EGF was a significantly stronger inducer of the myofibroblast phenotype than TGF-β and bFGF. α-SMA expression after treatment with TGF-β and bFGF was lower than with single TGF-β treatment.

Treatment with PDGF-AA, PDGF-BB, PDGF-AB, IGF-1, and IGF-2 showed no effect on α-SMA expression (data not shown).

FIG. 5 shows the results of double immunocytochemical staining of hDMC1.1 for Ki67 and α-SMA after treatment with TGF-β, EGF, bFGF, and their combinations. Only TGF-β and the combination of TGF-β and EGF resulted in significant upregulation of α-SMA. Treatment with EGF, bFGF, or the combinations (a) bFGF and EGF, (b) TGF-β and bFGF, and (c) TGF-β, EGF, and bFGF did not significantly influence α-SMA expression.

Invasion markers

FIG. 6 shows the expression of N-cadherin after polypeptide factor treatment. TGF-β and all combination treatments that included TGF-β upregulated N-cadherin expression.

FIG. 8 shows, individually, the expression of TNC and Ki67 staining in hDMC1.1 after polypeptide factor treatment, as well as the double immunostaining for these markers. TGF-β and its combinations but not EGF and bFGF alone or the combination of EGF and bFGF upregulated
TABLE 2. PROLIFERATION INDEX OF HUMAN DERMAL MESENCHYMAL CELLS (HDMC1.1) AFTER GROWTH FACTOR TREATMENT

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Treatment</th>
<th>Proliferation Index (%)</th>
<th>Standard Deviation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hDMC1.1</td>
<td>Control</td>
<td>28.80</td>
<td>3.50</td>
</tr>
<tr>
<td></td>
<td>TGF-β</td>
<td>29.66</td>
<td>7.30</td>
</tr>
<tr>
<td></td>
<td>bFGF</td>
<td>61.56</td>
<td>6.70</td>
</tr>
<tr>
<td></td>
<td>TGF-β + bFGF</td>
<td>73.20</td>
<td>4.60</td>
</tr>
<tr>
<td></td>
<td>TGF-β + EGF</td>
<td>47.24</td>
<td>3.70</td>
</tr>
<tr>
<td></td>
<td>TGF-β + EGF + bFGF</td>
<td>74.08</td>
<td>9.60</td>
</tr>
</tbody>
</table>

TGF-β, transforming growth factor-beta; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor.

DISCUSSION

The morphology and strong positivity for PH4 with a low expression of α-SMA in hDMC1.1 are consistent with our earlier report on this fibroblastic phenotype. Monoclonal anti-Ki67 antibody has been shown to be a strong indicator of cell proliferation by reacting with a nuclear antigen present during the G1, S, and G2 phases of mitosis but not in the G0 or resting phase and confirmed basal proliferation in the cell isolates.

hDMC1.1 expressed the polypeptide receptors, as shown using RT-PCR. The additional amplification products observed in FGFR-2 amplification can be explained by the local diversity exhibited by FGFR-2 due to alternative splicing of its messenger RNA. Gilbert et al. characterized 2 alternative FGFR-2 gene products, namely keratinocyte growth factor receptor and bacterially expressed

FIG. 6. Correlation between proliferation and differentiation in human dermal mesenchymal cell (hDMC1.1) immunostaining using anti-Ki67 and anti-smooth muscle α-actin (α-SMA) is shown in the first and second columns. The third column shows a combination of anti-Ki67 and anti-α-SMA in combination with 4′, 6-diamino-2-phenylindole. Scale bar = 50 μm. TGF-β, transforming growth factor-beta; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor.

FIG. 7. Expression of N-cadherin in human dermal mesenchymal cells. Total cell lysates were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot analysis with immunostaining for N-cadherin and tubulin. TGF-β, transforming growth factor-beta; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor.
kinase isoforms that are identical except for a 49-amino acid sequence in the extracellular portion. The latter authors have suggested that control of these alternative splice sites involves transacting factors.

The extra band observed using EGFR amplification could represent an alternative isoform, namely "soluble EGFR" as described by Reiter and Maibohle. The selected primers are capable of amplifying a truncated form of EGFR, as evidenced using Basic Local Alignment Search Tool (BLAST), by using an alternative polyadenylation signal.

PDGF-α and PDGF-β exhibit sequence homology, whereby the primer pair used is capable of amplifying both receptors with the same efficiency (E = 0.029, BLAST). Such an amplification would result in a doublet of bands, as observed for PDGF-β, with the upper band representing the amplification product of PDGF-α and the lower, that of PDGF-β.

TGF-β treatment did not result in stimulation of cell proliferation, as evidenced by optical density measurements. TGF-β is a known fibroblast trans-differentiating cytokine, which we have previously reported to result in a 100% trans-differentiation of hDMC1.11 The failure of TGF-β to stimulate proliferation is consistent with the reported observation that differentiation and proliferation are mutually exclusive phenomena made by Grotendorst et al.13 These authors hypothesized that the amount of energy used to drive either of these processes limits the energy available to drive the other. In other words, a 100% stimulation of cell toward either of these processes would result in no stimulation of the other. From our current observations, in the presence of TGF-β alone, hDMC1.1 did not proliferate but trans-differentiated, confirming preferential differentiation. Cells treated with TGF-β and EGF or bFGF showed significantly greater optical density and hence proliferation than those treated with TGF-β alone. Moreover the combination of TGF-β and bFGF indicated more proliferation than that of TGF-β and EGF and suggest exclusive proliferation.

The stimulation of proliferation by the combination of TGF-β and EGF was less than that observed for EGF and suggests an inhibitory effect of TGF-β on EGF-stimulated proliferation. The addition of bFGF to the TGF-β/EGF combination resulted in a significant net proliferation, indicating that bFGF was able to reduce the inhibition of proliferation imparted by TGF-β on these colonies.

The trans-differentiation of fibroblasts to a myofibroblast phenotype results in an upregulation of α-SMA. The combination treatment of TGF-β and EGF was a stronger inducer of the myofibroblast phenotype than TGF-β and bFGF. These findings are in keeping with those of Maltseva et al,17 who demonstrated an antagonistic relationship between bFGF and TGF-β. Moreover, the lower expression of α-SMA when bFGF was combined with TGF-β as opposed to TGF-β alone indicates possible dedifferentiation of myofibroblasts in the treated colonies.

Double immunostaining of treated colonies confirmed the Western blot findings that only TGF-β and the combination of TGF-β and EGF are capable of significantly inducing the myofibroblast phenotype. However, the proliferative effect of the TGF-β/EGF combination, as evidenced using Ki67 staining, was lower than that of EGF treatment alone. The latter indicated that, in the presence of TGF-β, the number of cells that proliferated was reduced, whereas others trans-differentiated. In comparison, the combination of TGF-β and bFGF failed to induce significant trans-differentiation but showed greater proliferation than single bFGF. As such, bFGF was capable of overcoming the inhibitory effects of TGF-β observed in the combination TGF-β/EGF. Moreover, the addition of bFGF to the TGF-β/EGF combination inhibited TGF-β-induced proliferation.
trans-differentiation while maintaining proliferation. These observations confirmed the mutual exclusivity of differentiation and proliferation.

TNC is a disulfide-linked, hexameric matricellular glycoprotein that is prominent in embryonic and adult tissues that are actively remodeling, such as in wound healing and in cancer, where invading myofibroblasts often express it. When present in mixed substrata, TNC may antagonize the pro-adhesive activities of other matrix proteins, such as collagen type I and as such can modulate invasion of myofibroblasts. Cadherins are transmembrane glycoproteins linked to the actin cytoskeleton via the catenins (α, β, and γ-catenin). N-cadherin is a pathfinding, pro-migratory molecule expressed in fibroblasts, myofibroblasts, neurons, smooth muscle, and endothelial cells. N-cadherin can interact with collagen and fibronectin of the extracellular matrix and function as an adhesive ligand. Moreover, the presence of N-cadherin at cell-cell borders allows inter-action via adherens junctions, the modulation of which can influence cellular migration and its direction. TGF-β and its combination with EGF and bFGF or with both upregulated N-cadherin and TNC.

We have demonstrated that the cocktail of EGF, bFGF, and TGF-β can promote the proliferation and invasion of hDMC1.1. TGF-β induced the expression of the invasion markers TNC and N-cadherin. EGF and bFGF are potent inducers of hDMC1.1 proliferation. In the combination treatment of EGF, bFGF, and TGF-β, bFGF is capable of overcoming TGF-β inhibition while exclusively promoting proliferation. The triple combination of EGF, bFGF, and TGF-β is a potentially useful tool in the modulation of fibroblast proliferation, differentiation, and invasion in tissue valve engineering.

Finally, as a note of caution, it should be emphasized that TGF-β1 and its isoform TGF-β2 have been implicated in fibrotic disease states. In addition to keloid formation in wound healing, an upregulation of TGF-β1 expression has been associated with fibrotic diseases, including that of the kidney and liver. Transgenic mice with elevated plasma levels of TGF-β1 have been reported to develop renal and hepatic fibrosis. The systemic administration of TGF-β2 in 1 clinical trial involving human patients suffering from multiple sclerosis was reported to be associated with reversible renal insufficiency. However, the transgenic mice and human trials referred to above involve high plasma levels of TGF-β, which are not indicative of the low concentrations of TGF-β applied locally, as in this study. Moreover, it is known that the duration of extracellular signals may influence cellular activity. In PC12 cells, for instance, it has been shown that cells can exhibit differentiation or proliferation responses to activation of extracellular signal-regulated kinases depending on the duration of the extracellular signal.

In the present study, after initial priming of cells, growth factors are withdrawn, and the primed cells can then be placed in a normal physiological environment in vivo, as opposed to the pathological situations referred to above, in which TGF-β1 signaling is sustained. As such, these reports do not establish a relation between transient, localized application of low concentrations of TGF-β and fibrosis. Nevertheless, despite the foregoing, the question of whether heart valves engineered with growth factors suggested in this study will develop fibrosis can only be answered unambiguously using in vivo animal studies, which are underway in our laboratory but are beyond the scope of the present study.

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Part IV

In Vivo Evaluation of Kangaroo and Porcine Xenogenic Scaffolds

In attempts to tissue engineer a functional aortic valve, matrices are currently implanted either after in vitro seeding of cells or un-seeded with expected repopulation in vivo. Most commonly, scaffolds are seeded with vascular interstitial cells. However, the parameters necessary to establish a pheno-typically appropriate cell population and density distribution are not fully defined [1]. As such whether a valve is sufficiently repopulated in vitro to regenerate the template scaffold is difficult to establish, as is the length of time necessary to accomplish the requisite repopulation. To date reports of implantation of matrices in sheep either seeded with cells prior to implantation or un-seeded with expected repopulation in vivo, have shown that matrices failed to repopulated and instead elicited a foreign body reaction [2].

Implanted matrices are expected to remain vital, be regenerated and thus not calcify. Such regeneration is not instantaneous and would require time to accomplish. If implanted un-seeded regeneration will have to await repopulation in vivo. Whether or not such a tissue engineered valve will be sufficiently endowed to prevent calcification of its components in this time frame is unknown. In fact, in light of the reported foreign body reaction this does not seem to be the case. Matrices exposed to the immune system can elicit a foreign body reaction and calcify. Current approaches to overcome these obstacles include attempts to seed xenogenic scaffolds with autologous endothelial cells and thus shield them from the host’s immune system. However it is known that complete covering of the scaffolds by endothelial cells can be variably complete and cannot be guaranteed. As in the case of bioprosthetic valves, other factors such as mechanical fatigue might be expected to contribute to the calcification of the scaffold as long as it is not renewed. Consequently, the calcification potential of matrices can have a significant impact. In this chapter we describe the evaluation of the calcification potential of both kangaroo and porcine a-cellular and non endotheliazed matrices in sheep compared to a contemporary bioprosthesis in order to assess the severity of this problem.
References


Chapter 7
Toronto SPV Valves Calcify Less Than Porcine and Kangaroo Aortic Valve Scaffolds In Sheep

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Abstract

Currently available bioprosthetic heart valves have a limited durability whereas their more durable mechanical counterparts require anticoagulant therapy. An engineered aortic valve would theoretically be capable of growth and like a normal native aortic valve it would maintain its durability by regenerating its extracellular matrix and require no anticoagulation therapy in recipients. A major limitation of currently available bioprosthetic valves is their propensity to calcify. At present, one approach to tissue engineering uses decellularized, xenogenic scaffolds that are implanted, either after in vitro seeding of cells or unseeded, with the expectation of complete matrix repopulation in vivo. Whether or not such a tissue engineered valve will be sufficiently endowed to prevent calcification of its components is unknown and as such the inherent calcification potential of the scaffold can be a significant determinant of its durability. This study examines the calcification potential of xenogenic biological scaffolds from two species, namely pigs (n=3) and kangaroos (n=3) in the sheep model and compared them to a commercially available glutaraldehyde treated porcine bioprosthetic valve (Toronto SPV®) which is a routinely used valve (n=3). Valves and matrices were explanted after 120 days, examined histologically (H&E and Von Kossa stain) and electron microscopically and their calcium content determined quantitatively by Inductively Coupled Plasma Optical Emission Spectrometry. Mean calcium content in milligram per gram of dry weight (mg/g-dw) of Toronto SPV valves was 2.63 mg/g-dw compared to 105.08 mg/g-dw in porcine and 43.81mg/g-dw in kangaroo matrices. Calcific deposits were located between as well as in close association with fibers of the collagen matrix in all tissue. Toronto SPV® valves calcified significantly less than the tested biological matrices irrespective of species of origin. Xenogenic scaffolds are inherently prone to calcification with a possible immune component.

Key Words

Kangaroo Aortic Valve Scaffold, Porcine Aortic Valve Scaffold, Toronto SPV Valves, Aortic Scaffold Calcification.
Introduction

The application of tissue engineering to cardiac valves attempts to produce a living organ comparable to the native valve both structurally and functionally and is a promising option in the treatment of aortic valve disease. An engineered aortic valve, for instance, would be capable of growth and like normal native aortic valves it would maintain its durability by regenerating its extracellular matrix and require no anticoagulation therapy in recipients. At present, two types of prosthetic heart valves are available, namely, bioprosthetic valves made from biological material and mechanical valves made from non biological material. Both types of devices are associated with significant limitations including a need for anticoagulation and degeneration [1-6]. Bioprosthetic valves include human homografts, and valves produced from either bovine pericardium or porcine aortic valves. Homografts are usually cryopreserved before use, while the latter two are usually chemically cross-linked using low concentrations glutaraldehyde [7]. Glutaraldehyde treatment of porcine aortic valves for human implantation was first introduced in 1969 [8] and the majority of bioprosthetic valves implanted currently are covalently cross-linked with glutaraldehyde. The Toronto SPV valve represents the latest generation of glutaraldehyde treated bioprosthetic heart valves manufactured from porcine aortic valves. Unlike mechanical prosthetic valves which have life long durability, bioprostheses do not require life long anticoagulation, but are limited in their durability. The limited durability of glutaraldehyde fixed bioprosthetic valves has been attributed to altered mechanical properties, antigenic properties of the cells, glutaraldehyde interactions and the calcification potential of cell membrane [9, 10, 11, 12].

Current approaches to tissue valve engineering include both the use of decellularized porcine xenogenic tissue and bioresorbable synthetic scaffolds [13] which are either seeded with cells in vitro before implantation or implanted unseeded, with expected in vivo repopulation by host cells [13, 14]. Acellular biological matrices are devoid of cells and theoretically do not harbour the potential of calcification due to cellular material as do currently available bioprosthetic valves. Moreover, acellular matrices, if not treated with glutaraldehyde would avoid glutaraldehyde interactions implicated in calcification.

Tissue engineered heart valves constructed from xenogenic tissue have already been implanted in humans [14, 15]. The results of these clinical implants however, have been catastrophic for patients due to valve failure and emphasizes the need to fully understand the pattern of failure of these constructs. [13, 15, 16]. In presently used bioprosthetic valves, calcific deterioration is a major cause of valve failure [17, 18]. While a functional tissue engineered valve is expected to remain vital
and regenerate its matrix and thus not calcify, such regeneration is not instantaneous and would require time to be fully accomplished in vivo. In this time frame, the intrinsic calcification potential of xenogenic scaffolds could significantly influence the construct’s calcification and hence its ultimate durability.

Current bioprosthetic valves have a life span of 15 to 20 years with negligible early complications. Their pattern of failure is well established, highly predictable and can be managed. The clinical feasibility of any tissue engineered valve will ultimately have to be judged against these criteria [19, 20].

Glutaraldehyde treated kangaroo aortic valves have been shown by us as well as others to have a calcification potential that is comparable to, if not lower than that of porcine aortic valves [21, 22]. More recently, we have reported on the hydrodynamic evaluation of kangaroo acellular matrices which suggests superior hydrodynamic properties compared to porcine matrices [23]. Consequently, kangaroo aortic valves might be considered a potential alternative source of acellular matrices for tissue valve engineering.

The aim of this study was to compare the inherent calcification potential of xenogenic matrices from two species, namely kangaroo and pigs, to that of a routinely used glutaraldehyde treated porcine bioprostheses, the TorontoSPV valve.

1. Materials and Methods

1.1 Valve Procurement and Scaffold Preparation

Three Toronto SPV valves of 21mm internal diameter used in this study were generously donated by ST. Jude Medical Corporation (St. Jude Medical, St. Paul, MN, USA). Porcine valves were obtained from the experimental slaughter house of the Department of Animal Production, Ghent University. The genotype of all animals was a cross between a hybrid sow and piétrain boar. Immediately after slaughter, hearts were retrieved and placed on wet ice for transportation to the Laboratory of Experimental Cardiac Surgery, University Hospital Ghent. Aortic valves were dissected free as four centimeter long conduits with a two millimeter rim of myocardium and stored in a cold preserving solution of isotonic saline as described below. Aortic valves were obtained from Eastern Gray Kangaroos (Macropus Giganteus) under licence in Queensland, Australia. Valves were procured at the Queensland Heart Valve Bank, University of Queensland, under similar conditions. Two additional decellularized valves of each type (kangaroo and porcine) were used to check the effectiveness of the decellularization procedure. A non-decellularized porcine
aortic valve was used to illustrate the relative cellularity of leaflets in porcine valves used in the manufacture of bioprostheses and for histological illustration of calcium in an unimplanted porcine leaflet. It should be emphasized that financial constraints precluded the use of Toronto SPV leaflets for the preimplant H&E and Von Kossa staining. Hence, for purely illustrative purposes non-decellularized native porcine leaflets were used.

While there is no universally standardized decellularization method, acellular matrices are typically prepared by cell lysis in hyper- and hypotonic solutions, with subsequent enzymatic digestion and detergent extraction [13, 24]. For purposes of this study, matrices were prepared using a patented detergent-enzymatic protocol which has been successfully used and reported in the literature by us and others [23, 24, 25, 26].

Essentially tissues were rinsed in hypotonic saline containing phenyl-methylsulfonylfluoride (PMSF: 1µM, SIGMA, Bornem) and antibiotics (streptomycin: 100µl/l; penicillin: 100µl/l mixture; SIGMA, Bornem, Belgium). Cells were then ruptured by subjecting the tissues to alternating treatments in hypotonic Tris-buffer (pH 8.0) and hypertonic Triton X solution (pH 8.0) (Biorad, Eke, Belgium). All solutions were supplemented with PMSF (1µM), penicillin/streptomycin solution (100µl/l respectively) and 50 µM butylated hydroxyanisole (SIGMA, Bornem, Belgium). After thorough rinsing, tissues were subjected to a digestive procedure using an enzymatic solution containing Dnase1, RnaseA, trypsin and phospholipases A2, C, and D (SIGMA, Bornem, Belgium). All steps were performed at 4°C.

1.2 Implantation and Explantation of Valve and Matrices

The study was approved by the ethical commission for animal experiments, Ghent University. All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health NIH (publication No.85-23, revised 1985). Nine juvenile, suffolk sheep used in this study were obtained from a licenced supplier in Belgium.

The anaesthetic and operative procedure employed during right sided heart bypass and pulmonary valve implantation in sheep has been reported by us in detail elsewhere [27]. In short, the sheep were premedicated intravenously with 0.1 mg/kg midazolam (Dormicum®, N.V. Roche S.A., Brussels, Belgium) and 0.1 mg/kg methadone (Mephenon®, N.V. Demolin S.A., Brussels, Belgium). Anaesthesia was induced with 2-4 mg/kg propofol (Diprivan®, Astra Zeneca, Destelbergen, Belgium) and maintained with isoflurane (Isoflo®, Abbott Laboratories Ltd., Queenborough, Kent, United Kingdom) in oxygen, combined with infusions of propofol and fenta-
nyl (Fentanyl-Janssen®, Janssen-Cilag, Berchem, Belgium). The heart was exposed by a left anterolateral thoracotomy via the third intercostal space. Systemic anticoagulation was induced with 3 mg/kg heparin (Heparine Leo®, Leo Pharma N.V./S.A., Zaventem, Belgium). Right heart bypass was established by pulmonary and right atrial cannulation. The heart was kept normothermic and beating throughout the procedure. The pulmonary artery was clamped, transected and the test valves and matrices were interposed in the pulmonary trunk using running Proline 5.0 sutures (Eticon, Merelbeke, Belgium) distally and proximally. Through a separate, lower incision in the pulmonary artery, the native pulmonary valve was rendered incompetent by destruction of its leaflets. Animals were weaned from bypass and heparine was neutralised with 3 mg/kg protamine. An intercostal block using 0.5 % bupivacaine (+ epinephrine) (Marcaine®, Astra Pharmaceuticals N.V./S.A., Brussels, Belgium) was installed before closing the chest with a temporary thorax drainage system in place. Post operatively and during the first three post operative days, analgesics included 0.1 mg/kg methadone IM every four hours during daytime and 0.01 mg/kg buprenorphine IM (Temgesic®, Schering-Plough N.V./S.A., Brussels, Belgium) in the evening. Animals were euthanized after 120 days with an intravenous bolus of 50 mg/kg pentobarbital (Natriumpentobarbital®, Kela N.V., Hoogstraten, Belgium) and the valves explanted.

All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health NIH (publication No.85-23, revised 1985).

1.3 Explant Analysis

(a) Light Microscopy

Samples for histology were fixed in 4% phosphate buffered formaldehyde (Merck, Darmstadt, Germany) and embedded in paraffin. Five micron thick sections were cut and stained for calcium (Ca) with Von Kossa stain.

(b) Electron Microscopy

Samples were fixed in phosphate buffered solution of 4% formaldehyde (Merck, Darmstadt, Germany), supplemented with alcian blue to precipitate and preserve proteoglycans during the dehydration. Specimens were subsequently post fixed with 1% osmium tetroxide (OsO₄) in phosphate buffer (Merck, Darmstadt, Germany) and embedded in epoxy resin. Ultra-thin 60nm sections were cut and examined with a Jeol 1200 EX-II transmission electron microscope at 80 keV.
(c) Calcium content.

Samples were lyophilized and subsequently mineralized by ashing during 3 hours at 450 °C. Ashes were dissolved in six molar boiling nitric acid (Merck, Darmstadt, Germany) and Ca was estimated spectrometrically using ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometry (Varian, Mulgrave, Victoria, Australia). Calcium content is expressed as milligram per gram of tissue dry weight (mg/g-dw).

2. Results

Figure 1 illustrates a Toronto SPV® valve and kangaroo and porcine aortic matrices that were implanted. Hematoxylin-Eosin (H&E) stained histological preparations confirmed the decellularization of the kangaroo and porcine matrices as illustrated in figure 2a and 2b. The relative cellularity of a non decellularized porcine aortic valves representative of valves used to manufacture glutaraldehyde treated porcine bioprostheses, is illustrated in fig 2c. Kangaroo and porcine aortic matrices were rendered completely acellular following the decellularization procedure. Figure 3 illustrates an acellular matrix (kangaroo) implanted in the pulmonary position of the right ventricular outflow tract in a sheep. All explants showed some fibrous over growth extending from the left ventricle towards the leaflets.

Fig. 1. Illustration of a Toronto SPV bioprosthese and Kangaroo (K) and Porcine (P) matrices used in this study.
Fig. 2. H&E stain of porcine (a) and kangaroo (b) matrices and a native porcine aortic leaflet representative of that used in preparation of the Toronto SPV valve (c). Note the absence of cells in the matrices.

Fig. 3. An implanted matrix in the pulmonary position of the right ventricular outflow tract in sheep.

2.1 Von Kossa Stain

Figure 4 shows the von Kossa stain of explanted leaflets as well as of unimplanted matrices. Leaflets from the Toronto SPV valves showed significantly less staining compared to either type of matrix. Kangaroo matrices stained less intensely compared to porcine matrices. In the Toronto SPV explants, calcium deposits appeared to follow the distribution of cells and also occurred in larger isolated deposits. In matrices, calcific deposits were located throughout the leaflet and particularly at the free edges.
2.2 Electron Microscopy

Electron microscopic sections of explants are illustrated in Fig. 5. Calcific deposits were observed in all valves and matrices. In the Toronto SPV bioprostheses, calcification appeared to be associated with cells and showed a diffuse distribution. In the acellular matrices the calcific deposits were distributed throughout the leaflet in close association with the collagen matrix. In areas where larger deposits of calcium were found they were located between and in association with the collagen fibers. In one preparation in which elastin was observed in an acellular porcine matrix, calcific deposits were also strongly associated with the elastin fibers (Fig. 5C).
Figure 5. Electron microscopic sections of explants at high and low magnifications indicated by bar: Toronto SPV valve (A and B), Porcine matrix (C and D) and Kangaroo matrix (E and F). In C the dark structure represents elastin fibers.

2.3 Quantitative Calcium Determination

Table 1 shows the raw data and Fig. 6 illustrates the mean calcium content in milligram per gram of dry weight (mg/g-dw) for each type of tissue. The mean calcium content in Toronto SPV was lower (2.63mg/g-dw) compared to either kangaroo (43.8mg/g-dw, with p= 0.066) or porcine(105.8 mg/g-dw, with p= 0.001) acellular matrices. Statistical analysis was done using the student’s t-test, with a value of p<0.05 taken to be significant. Although the significance of the p value for comparison of means between the Toronto SPV and porcine matrices appear borderline, bearing in mind that the limited number of observations due to the small sample do not justify a normal distribution in the groups, it was clearly convincing.
3. Discussion

The most significant finding of this study is that xenogenic matrices calcified significantly more than the Toronto SPV valve, a routinely implanted glutaraldehyde treated porcine bioprosthesis, regardless of whether such matrices were derived from porcine or kangaroo aortic valves. To our knowledge this is the first study to compare the calcification potential of two xenogenic scaffolds to that of a routinely used porcine bioprosthesis in the sheep circulatory model.

We have previously reported the histological appearance of non-decellularized kangaroo and porcine aortic leaflet [21]. The light microscopic examination of both types of xenogenic matrices in this study confirmed the effectiveness of the decellularization procedure. Kangaroo and porcine matrices were rendered completely acellular.

Several factors have been implicated in the calcification of glutaraldehyde treated porcine bioprosthetic valves. In children, in whom bioprosthetic valve failure is

<table>
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<th>Mean Ca (mg/g-dw)</th>
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Table 1. Raw data of calcium content (Ca) in the different tissues. K = kangaroo, P = porcine, mg/g-dw = milligram/gram dry weight, Sd = standard deviation

Figure 6. Average calcium content in milligram per gram of dry weight (mg/g-dw) for each type of tissue.
observed more commonly than in adults a possible involvement of high serum phosphate and osteocalcin as well as enhanced metabolism of parathyroid hormone and vitamin D has been suggested [28, 29, 30]. Both the cellular component as well as glutaraldehyde treatment have been implicated in the pathological calcification in these prosthetic valves.

In the present study, we observed deposits of calcium in close association with the devitalized connective tissue cells as well as in close association with collagen and elastin fibers. Indeed phospholipids and phosphoserine containing proteins have been found in pathologically calcified tissue and products of cellular degradation appeared to be the early sites of calcification [31]. These observations confirm previous ultrastructural findings in explanted valves which implicated valvular cells in the calcification process [32].

While it is widely acknowledged that glutaraldehyde treatment results in a reduction of immunological recognition of the xenogenic tissue and its stabilization to degradative enzymes in humans [33], glutaraldehyde has been suggested to be a cause of pathological calcification [9, 34]. In bioprosthetic valves, glutaraldehyde introduces thermally and chemically stable cross-links which become compromised with time and result in a leaching out of glutaraldehyde [35]. Such free aldehydes have been shown to be easily oxidized to carboxylic acid which is a potential site for calcium binding [36, 37]. Glutaraldehyde also stiffens and alters the biomechanical characteristics of porcine valvular tissue. Such alterations are associated with demonstrable changes in leaflet motion which produce abnormal stress patterns causing buckling, accelerated calcification and eventual tissue failure [38]. In addition, glutaraldehyde cross-linked valves are and remain non viable tissues, without opportunity for either tissue renewal or growth which is explained by glutaraldehyde cytotoxicity and the inability of cells to penetrate the cross-linked matrix [35, 39]. Furthermore, although stable cross-links in cellular and extracellular matrix proteins are considered to reduce immunogenicity, porcine tissue retains a residual ability to trigger an immune response that can activate macrophages, which in turn can obtain an osteoblast calcium depositing phenotype [40]. Moreover, it is well established that cell and the cellular membrane provoke early calcification of biological heart valves, whereby a direct relation between specific antibody response and the calcification of porcine tissue has been demonstrated [12, 41].

Acellular xenogenic matrices such as those tested in this study are devoid of cells and are not treated with glutaraldehyde. As such these constructs might be expected to calcify less than a glutaraldehyde treated porcine bioprostheses such as the Toronto SPV valve. On the contrary, xenogenic matrices in this study demonstrated a greater propensity to calcify and suggest that their preparation by decellularization and
omission of glutaraldehyde treatment is insufficient to completely mitigate calcification.

Xenogenic scaffolds are known to be more proinflammatory when cells are disrupted and cell debris, cytokines, and other inflammatory moieties are not thoroughly removed from the matrix [42]. Recently, Reider et al. elegantly demonstrated residual immunogenicity in porcine xenogenic scaffolds which resulted in a greater stimulation of macrophage response compared to decellularized human aortic valves in an in vitro assay using the migratory response of U-937 cells, a human monoblastic cell line [43]. Indeed a failed tissue engineered porcine scaffold which was implanted in humans with catastrophic results and has been subsequently withdrawn from the market has been shown to possess gal-\(\alpha\)\(^1,3\) isotope, the major xenoantigen(s) recognised in pigs by human natural antibodies [15, 44]. In the latter studies an inflammatory macrophage and neutrophil granulocyte infiltration was reported in the explants. Also, studies on human homografts, have shown that calcification in these valves is related to a persistent immunoreactivity against donor antigens [45]. As such, in xenogenic scaffolds, regardless of their species of origin, in which immunogenic moieties persist, it is highly probable that the observed calcification in xenogenic matrices might be a manifestation of an immune response. Current approaches to tissue valve engineering include attempts to overcome these obstacles by seeding xenogenic or allogenic scaffolds with autologous endothelial cells before implantation and thus shielding them from the host’s immune system. However it is known that complete covering of the scaffolds by endothelial cells can be variably complete and cannot be guaranteed [47].

It should be emphasized that while xenogenic scaffolds did calcify more than Toronto SPV valves, there was a difference in the observed calcification in matrices themselves. Kangaroo matrices calcified less than porcine matrices. As discussed above, several factors have been implicated in tissue valve calcification. We have previously shown that kangaroo aortic valves differ histologically and hydrodynamically from porcine aortic valves [21, 23]. It is highly conceivable that interspecies differences may contribute to the observed differences in calcification of xenogenic matrices. Further investigation of the differences between kangaroo and porcine matrices is as such warranted. While such investigations were beyond the scope of the present study, current research in our laboratory is also addressing this issue.

In conclusion, Xenogenic scaffolds calcify more than the glutaraldehyde treated Toronto SPV\(^\circledR\) porcine bioprosthesis valve despite their decellularization and the absence of glutaraldehyde treatment. Tissue engineered scaffolds will need to outperform current bioprostheses in their calcification potential in order to take their
place in the treatment of heart valve disease. The immunogenicity of such scaffolds might help to explain their calcification and further research into rendering matrices immunologically inert is warranted. Further research at our laboratory is dedicated to improving biological scaffolds as well as fully characterizing associated fibrotic development.

References


44. Sandrin MS, McKenzie IF, Gal alpha (1,3) Gal, the major xenoantigen (s) recognized in pigs by human natural antibodies. Immunol rev 1994; 141: 169-190.


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General Discussion

The aim of this study was to initiate a research programme in the tissue engineering of a functional aortic valve by addressing several fundamental issues considered critical in heart valve engineering.

This study demonstrated that a-cellular biological scaffolds can be prepared from biological valves using a contemporary decellularization procedure and confirmed similar reports in the literature [1,2]. Decellularization by definition alters the material properties of the matrix and may weaken tissues. Such effects were evaluated and suggest that the procedure employed did not significantly alter the mechanical strength of the tissue but significantly reduced their proteoglycan content. Although these results are in keeping with the literature it should be cautioned that decellularization results in significant histological changes whereby tissues become highly porous and exhibit locally collapsed microstructure [3,4]. The effects of these changes might not always be appropriately reflected by present methods of testing. The use of porcine xenogenic scaffolds currently dominates investigations in tissue valve engineering. In this study we opted for biological matrices, for although polymer based scaffolds are attractive from an engineering perspective in that they can be created in a variety of shapes and sizes, they will still need to replicate the performance characteristics of the native aortic valve. Moreover they must successfully withstand the inevitable foreign body response after implantation. Biological scaffolds are structurally and morphologically more similar to the native aortic valve and eliminate the design difficulties faced by a polymer scaffold.

In this study we have introduced a novel biological scaffold for use in tissue valve engineering, namely kangaroo xenogenic matrices. Kangaroo aortic valves differ histologically and morphologically from porcine valves. Importantly they do not have a rim of muscle in the right coronary cusp as is the case in porcine valves and they are thinner than porcine valves [5,6]. A muscular bar can be obstructive to the flow of blood through a valve. We have presented hydrodynamic finding in this study showing that the effective orifice area and performance index of kangaroo matrices are larger than those in equivalent porcine matrices.

The study also addressed the logistics of tissue valve engineering, in particular storage and availability of matrices. Decellularization procedures that take several days are not uncommon [3]. The protocol used in this study took five days and could be problematic when a valve is needed on short notice. Consequently, we report for the
first time, the biomechanical effects of cryopreservation on xenogenic matrices by evaluating them before and after cryopreservation. Cryopreservation did not significantly influence the strength of matrices but cryopreserved matrices showed significantly higher strain compared to non-cryopreserved matrices when subjected to the same force. Of course, it is assumed that a matrix will be repopulated and regenerated within a certain time frame in an engineered valve. Whether the altered mechanical properties will be sufficient to sustain the matrix during this time is unclear to us. Based on the results of this study cryopreservation might not be appropriate for storing a-cellular matrices.

Porcine cells express gal-α1,3 isotope, the major xeno-antigen(s) recognised in pigs by human natural antibodies [7]. A-vital cells in current bioprosthetic valves are widely acknowledged to contribute to their calcific deterioration. A-cellular porcine matrices are per definition devoid of cells. Nevertheless, when implanted in the sheep model, a-cellular xenogenic matrices retained the propensity to calcify regardless of whether they were of porcine or kangaroo origin. Indeed, we observed more calcification in matrices than in a contemporary bioprosthetic valves. Recent evidence [8] suggest that xenogenic scaffolds retain their immunogenicity. In this case, current decellularization processes may not be sufficiently effective in removing immunogenic material. Current investigations by our laboratory are aimed at optimising matrices by addressing their immunogenicity.

The study also presented a model for obtaining high densities of myofibroblasts for tissue valve engineering from both arterial and dermal mesenchymal cells using TGF-β1. TGF-β1, successfully trans-differentiated arterial and dermal fibroblast yielding larger densities of myofibroblasts for cell seeding. However, TGF-β1 may also inhibit the proliferation of fibroblasts within a leaflet. The cocktail of EGF, bFGf and TGF-β can promote the proliferation and invasion of fibroblasts. TGF-b, induced the expression of the invasion markers tenacin-C and N-caderine. EGF and bFGF are potent inducers of proliferation. In the combination treatment of EGF, bFGF and TGF-β, bFGF was capable of overcoming TGF-b inhibition, while exclusively promoting proliferation. The triple combination EGF, bFGF and TGF-β is currently being used in our recellularization protocol. Numerous studies have indicated that a bioreactor environment, which simulates hemodynamic conditions, may be advantageous in the in vitro seeding of constructs [9,10]. In addition to seeding matrices under static conditions, present investigations in our department also make use of a custom made bioreactor for dynamic seeding in which matrices are placed after they are mounted on a cylindrical support. [Fig 10a and 10b].
Fig. 10. A mounted matrix (a). Temperature controlled Bioreactor for cell seeding under physiologic hydrodynamic conditions (b).
References


7. Sandrin MS, McKenzie IF, Gal alpha (1,3)Gal, the major xenoantigen(s) recognised in pigs by human natural antibodies. Immunol Rev 1994; 141: 169-190.


Future Perspectives

Although a tissue engineered heart valve is a promising concept, its production is not around the corner. Criteria as to what per definition constitutes a functional tissue engineered valve are currently lacking and the definition of tissue engineering at the beginning of this manuscript, while encompassing is not tissue specific.

Several questions persist:

– is an engineered construct supporting metabolizing cells a functional valve?
– to what extent is a tri-laminar structure required?
– how to test durability?
– how to guarantee vitality in vivo?
– what are the markers?

The lack of standards for judging constructs may have contributed, at least partly, to the fact that successes in tissue valve engineering were being claimed in several areas and even the American Heart Association Web site has an article entitled “Tissue Engineered valves give diseased hearts new life [1].” Despite the hype, we do not as yet have the technology to guarantee a satisfactorily functioning valve. A recent report with catastrophic consequences for patients emphasizes this point [2]. The valve involved in the latter report was a tissue engineered valve produced by the commercial enterprise CryoLife Inc., USA and named the Cryolife Synergraft®. It was produced from decellularized porcine valves and was implanted in pediatric patients in Austria without reported long term animal studies, after receiving CE approval in 2000 [3]. Some valves failed only days after implantation and deaths were reported after 7 days (patient aged 7 years), 6 weeks (patient aged 9 years) and one year (patient aged 25 years). In another patient the valve was preventatively removed after 2 days. Progress is indeed slow but we need to be patient for while science might not always be successful, patients are always first.

References


Summary

This study addresses some fundamental issues involved in tissue engineering an aortic valve. A tissue-engineered valve developed using three-dimensional synthetic or biological scaffolds, which are repopulated with autologous cells, offers an opportunity to overcome the limitations of current prosthetic valves. Current prosthetic valves include mechanical, bioprosthetic and human homograft valves and they are associated with major limitations. Mechanical valves are durable but require lifelong anticoagulation. Bioprosthetic valves and homografts avoid anticoagulation but are a-vital with limited durability. A vital tissue engineered valve would require no anticoagulation, utilize the body’s mechanisms for repair and re-modelling and have the potential to grow. There is as yet, no functional tissue engineered valve available. Tissue valve engineering is an evolving field of research, which faces several critical issues, which include scaffold and cell selection as well as appropriate conditions to accomplish repopulation.

Biological scaffolds were prepared from kangaroo and porcine aortic valves using a detergent – enzymatic procedure. The logistics of having readily available biological for use on short notice as in a clinical setting was addressed by investigating the feasibility of cryopreserving matrices. Matrix biomechanical properties were evaluated by determination of collagen and proteoglycan content and a burst test. The hydrodynamic properties of both types of matrices were evaluated using a left heart model and their calcification potential in the sheep model. Myofibroblasts, the major phenotype of valvular interstitial cells, were obtained by establishing a model for arterial and dermal fibroblast transdifferentiation by TGF-β1. Modulation of the proliferation and invasion of myofibroblasts in matrix repopulation was investigated with a combination of growth factors including EGF, bFGF as well as TGF-β1. Biological scaffolds can be procured from porcine and kangaroo aortic with preservation of tissue strength but without eliminating their calcification potential. Kangaroo matrices showed superior hydrodynamics compared to porcine matrices. TGF-β1 transdifferentiates fibroblasts into active myofibroblasts. A particular cocktail of growth factors can positively influence myofibroblast proliferation and invasion into the matrix.

In deze studie werden biologische matrices ontwikkeld uit varkens of kangoeroe kleppen na behandeling met een enzymatische detergent oplossing. De reden om over te gaan op het gebruik van deze biologische matrices was gebaseerd op de reeds lang bestaande kennis van de cryo-preserveerde homografts in de kliniek. De karakteristieken van deze matrices werden biochemisch getest door bepaling van het collageen en de proteoglycans. Ook werd de weerstand mechanisch getest. De hydrodynamische kenmerken werden nagegaan in een linker hart testmodel en het potentieel tot calcificatie, de achillespees van alle bioprothesen, werd getest in het klassieke schaapmodel. Myofibroblasten, het belangrijkste fenotype valvulaire interstitiële cellen, werden verkregen door transdifferentiatie van humane en schaap arteriële en dermale fibroblasten na toedienen van TGF-β. De matrices herbezaaien door middel van proliferatie en invasie van de myofibroblasten werd bekomen door het toedienen van de groeifactoren EGF, bFGF in combinatie met TGF-β1.

Biologische matrices kunnen worden bekomen uit kangoeroe en porcien materiaal met behoud van hun inherente sterkte doch zonder hun calcificatie potentieel te neutraliseren. Kangoeroe matrices zijn hemodynamisch superieur ten opzichte van varkenskleppen. De juiste combinatie van groeifactoren maakt het mogelijk om deze myofibroblasten de matrix te laten binnendringen en uiteindelijk te herbezaaien.
The world of the senses is real, but it must be known for what it is: unity appearing as multiplicity.

(The Upanishads)