

Review Article: Tissue Engineering of Semilunar Heart Valves: Current Status and Future Developments

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Heart valve replacement represents the most common surgical therapy for end-stage valvular heart diseases. One major drawback that all heart valve replacements have in common is the lack of growth, repair, and remodeling capability once implanted into the body. The emerging field of tissue engineering is focusing on the in-vitro generation of functional, living semilunar heart valve replacements. This review presents a state-of-the-art overview of

the physiological and biomechanical requirements of semilunar heart valves, focusing on the aortic valve. Moreover, recent heart valve tissue engineering is summarized and future options and improvements on the way towards clinical applications are discussed.

The Journal of Heart Valve Disease 2004;13:272-280

Development and morphology of aortic valve leaflets

When the first heart contraction takes place in embryonic development, the heart is no more than a tube consisting of a single lumen. This tube is transformed into an H-shaped outflow channel with large tissue cushions in the right and left outflow tracts. These will each divide into three equal mounds of cushion material and form the origins of the aortic and pulmonary valve (1,2). Endothelial cells lining these cushions appear to be able to differentiate into leaflet interstitial cells, regulated by local growth factors (3).

According to Maron and Hutchins (1), the hemodynamic environment during development of the valve cusps determines the cell shape, proliferation and fiber formation. The cells on the ventricular side of the leaflets are flattened, due to the shearing effect of the blood flow during ventricular ejection, whereas the cells at the arterial side remain more cuboidal. The cusps grow by proliferation of cells in the downstream end, the region with low pressure and low shearing force. Cell proliferation seems to stop when the leaflets are long enough to contact the arterial wall above the sinuses during opening. Elastic fibers become promi-

nent at the ventricular side of the leaflet, which is exposed to intermittent flexural stresses during systole. At the arterial side, which is exposed to the predominantly static stresses during diastole, collagenous fibers develop. At the line of closure, the leaflets consist of solely collagenous fibers, which correlate to the tensions at both sides of the leaflets.

The development of the aortic valve takes place under pressure values below 10 mmHg, at heart rates ranging from 65 to 160 beats per minute, and hypoxic conditions (2). The acceleration of the heart rate might be a compensatory phenomenon in the absence of the Frank-Starling mechanism, as the immature fetal myocardium does not possess the ability to increase the ejection fraction in response to increasing preload. By increasing hematocrit and a shift of the hemoglobin-oxygen dissociation curve towards optimized oxygen binding characteristics, the fetus compensates for the hypoxic conditions.

The load-bearing part of adult aortic valve leaflets shows a layered architecture within the endothelial coverings (Fig. 1), enabling the extraordinary changes in shape and dimension to take place. The ventricularis - the layer at the inflow surface - is predominantly composed of radially aligned elastin fibers. The central layer, the spongiosa, consists of loosely arranged collagen and an abundant amount of proteoglycans. The layer at the outflow surface, the fibrosa, comprises mainly circumferentially aligned collagen fibers. All collagen bundles diverge into the aortic wall, thereby transferring the gross load on the leaflet to the aortic

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wall. The individual layers can easily compress and shear during opening and closing of the valve. The fibrosa and ventricularis are inherently preloaded due to their attachment to each other, the fibrosa under compression and the ventricularis under tension (4).

Among the valvular interstitial cells, three cellular phenotypes can be identified: (i) smooth muscle cells, arranged in bundles or just as single cells (5); (ii) fibroblasts maintaining the extracellular matrix, with 60% (6) of them being myofibroblasts; (iii) cells that have phenotypic features of both fibroblasts and smooth muscle cells (7,8). These cellular phenotypes are situated depending on their biological and mechanical microenvironment. Myofibroblasts and fibroblasts are able to convert from one to another, triggered by either a lack of mechanical tension or the presence of continuous mechanical tension (8). According to Della Rocca and colleagues (9), fibroblasts are mainly found in the ventricularis of human aortic valve leaflets, while myofibroblasts and smooth muscle cells are segregated in the fibrosa. A comparable distribution pattern can be found in the vascular wall with myofibroblasts and smooth muscle cells in the medial layer and fibroblasts in the adventitial layer. In the case of hypertension, fibroblasts of the adventitia convert into myofibroblasts to adapt to the higher mechanical loads (10). This same adaptation process might explain the distribution pattern of cellular phenotypes in the aortic valve leaflets. Due to the pressure difference over the leaflets, more smooth muscle like cells are expected in the fibrosa compared to the ventricularis. Pressure levels may influence differences between pulmonary valve leaflets and aortic valve leaflets as well (9). In disagreement with the pheno-

type distribution in the human aortic valve as described above (9), the phenotype distribution in porcine aortic valve leaflets was reported to be different, with smooth muscle cells and myofibroblasts more prevalent in the ventricularis than in the fibrosa (11).

The idea of passively functioning aortic valve leaflets was refuted by identifying a smooth muscle cell system in the leaflets (5), contractile properties of valvular interstitial cells (6,7,12), and sensory nerve elements in the leaflets (13). Contraction within the leaflets might help to sustain the hemodynamic forces that are exerted on the leaflet during systole and diastole (6), and represents a reactive cytoskeleton that can anchor collagen fibrils during valve closure (7).

Structure-function properties of aortic valve leaflets

The individual layers of valve leaflets show different mechanical characteristics due to their differences in composition (14-16). The fibrosa is considered to be the main load-bearing layer of the leaflet, and prevents excessive stretching (17). The difference in radial and circumferential extensibility - a phenomenon known as anisotropy - is not as large in this layer as it is in the ventricularis, where the radial extensibility is much greater than the circumferential extensibility (14-16). The overall mechanical response of the leaflet is a summation of the individual mechanics of the layers. Lo and Vesely (18) measured a maximal extensibility of porcine aortic valve leaflets of 24% in the radial direction and 11% in the circumferential direction by whole-valve biaxial testing; this is a reliable method of testing in which the natural biaxial loading environment in the valve is reflected. In the circumferential direction, the mechanical behavior exhibits the properties of collagen bundles, whilst in the radial direction the elastin mesh is the predominant factor. The leaflet shows an extremely low compressive modulus, which is most likely facilitated by the spongiosa (14).

Simply examining the function and structure of collagen does not explain the highly non-linear, viscoelastic stress-strain characteristics and anisotropic behavior of the valve cusps. Uniaxial tensile tests of circumferential and radial leaflet strips with and without digestion of elastin display a great impact of elastin on the stress-strain curves as shown by Vesely (4). Without elastin, the overall stiffness of the leaflets increases (19). This indicates that elastin is indeed a functional element in the aortic valve, and its role should not be underestimated due to its low content compared to collagen, being 13% versus 50% by dry weight respectively (4,19).

Elastin is not only limited to the ventricularis, but is present throughout the leaflets as a complex network of sheets, tubes, and fibers. It functions as a 'housekeep-

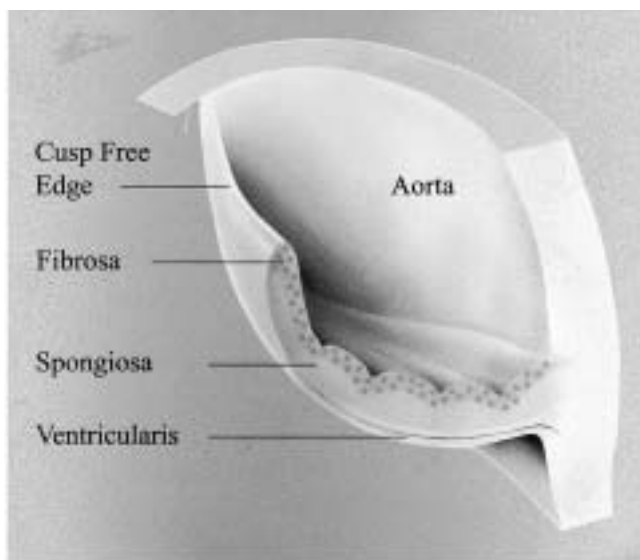


Figure 1: Configuration of the fibrosa, spongiosa, and ventricularis within the aortic valve leaflet.

er', restoring the collagen fiber geometry to its original configuration between loading cycles (4). In the fibrosa, a matrix of elastin surrounds the collagen bundles, storing energy as it becomes stretched during diastole. This energy is used to return the collagen to its original structure at systole. The elastin in the ventricularis consists of a large, very dense sheet. The size of the elastin layer is representative of the forces needed to hold the leaflet in its resting structure, and to allow the valve closure to progress smoothly to a point at which the collagen takes over the load (16). The mechanisms through which forces are transferred between collagen and elastin are not yet properly understood (15,16). Either elastin fibers connected between different collagen fibers help to return the collagen bundles to their crimped state, or elastin fibers attached to different parts of the same collagen fiber (15).

Schoen and Levy (20) summarized the biomechanics of the aortic valve as follows. When the valve is nearly closed and the collagen bundles in the fibrosa are fully unfolded, collagen is the load-bearing element, enabling a stress increase while preventing a prolapse of the leaflets. The loose spongiosa layer is able to dissipate the shock of closure of the leaflets, as the hydrophilic proteoglycans in this layer readily absorb water, and swell. Due to deformation of the sinus walls, which results in an increase in volume, the pressure difference across the valve decreases. During opening of the valve, elastin extends at minimal load in the ventricularis to return the fibrosa in its original corrugated state, facilitated by the spongiosa that dissipates the arising shear stresses.

Heart valve replacements

The most common treatment for end-stage valvular diseases is surgical replacement of the valve with either a mechanical or bioprosthetic valve. Mechanical heart valve replacements display good structural durability, but are associated with a risk of prosthetic valve endocarditis and high rates of thromboembolic complications caused by their non-physiological surface and flow abnormalities. Lifelong anticoagulation therapy is necessary in these patients, and this is associated with a substantial risk of spontaneous bleeding and embolism, particularly in patients aged over 70 years (21).

Bioprosthetic heart valve replacements are either of animal origin (xenografts), such as porcine aortic valves and bovine pericardial valves, or they may be taken from human donors (homografts). Xenografts are chemically crosslinked - this inhibits autolysis, enhances the mechanical stability, and creates the possibility of having valves of different size directly available. However, these valves differ in many respects from native valves, for example in their opening and

closing behavior due to the above-mentioned chemical pre-treatment (20). Explanted xenografts are shown to be stiffer in the radial direction and less stiff in the circumferential direction compared to native porcine valves (22). The risk of thromboembolic complications is much lower, but the valve's durability is limited. Structural failure is strongly age-dependent, making xenografts attractive for the elderly but less suitable for children and young adults (20).

One important aspect in xenotransplantation is the risk of zoonoses - human diseases caused by infectious agents from animals (23) - which might even be facilitated by the mandatory immunosuppression (24,25). The identification of porcine endogenous retroviruses and prionic diseases has given rise to great concern. Recently, epidemiological data have strongly indicated the transfer of Creutzfeldt-Jakob disease from cattle into humans either via infected meat, via surgical materials derived from bovine gut, or via drugs or vaccines prepared using fetal calf serum (26). Porcine endogenous retroviruses (PERV) may be present in all organs, as multiple copies of PERV can be integrated into germ-line DNA. New and more infectious groups of PERV are being identified (27), as well as their capacity to infect various types of human cells in vitro (28,29). There may be other infectious agents from animals that can be transferred into humans by xenotransplantation, though as yet their existence has not been proven.

Cryopreserved aortic donor valves are the heart valve replacements closest to the natural valve, as they are not thrombogenic and have a low risk of infection. They are not chemically crosslinked and exhibit good mechanical properties, which prolongs their lifetime (19). However, their disadvantages are their limited availability, a more difficult implantation technique (21), and failure associated with a specific immune response, especially in young children (30). The use of cryopreserved pulmonary homografts as aortic heart valve replacements has been shown to result in early failure as the fiber structure of the pulmonary valve is less resistant to the hemodynamic environment in the aortic position compared to cryopreserved aortic valves (31). Furthermore, these valves were shown to suffer from gross regurgitation in vitro, highlighting their unsuitability as aortic valve replacements (32). A controversial issue regarding cryopreserved homografts concerns the viability of the inherited endothelial and interstitial cells. A lack of viable cells after implantation was identified (20), as well as the long-term survival of cellular elements (33). In general, although cryopreservation reduces cellularity, the expression of strong allogeneic antigens can still be demonstrated, and this might trigger the immune system of the host, resulting in graft rejection (34).

Several attempts have been made to create functional

heart valve replacements, with the ability to grow, repair, and remodel, using the concept of tissue engineering (Fig. 2). In tissue engineering, the patient's own cells, isolated for example from a blood vessel and expanded using standard cell culture techniques, are seeded onto an appropriate carrier, termed the *scaffold*, in the shape of a heart valve. Subsequent stimulation, transmitted via the culture medium (biological stimuli) or via 'conditioning' of the tissue in a bioreactor (mechanical stimuli), promotes tissue development. An ideal scaffold must be at least 90% porous (35), and possess an interconnected pore network, as this is essential for cell growth, nutrient supply, and the removal of metabolic waste products. Besides being biocompatible, biodegradable, and reproducible, the scaffold should also display a cell-favorable surface chemistry and match the mechanical properties of the native tissue. The rate of degradation should be proportional to the rate of tissue formation, and also be controllable in order to ensure mechanical stability over time (36,37).

Tissue-engineered heart valves based on biological scaffolds

Donor heart valves or animal-derived valves depleted of cellular antigens, which makes them less immunogenic, can be used as a scaffold material. Removing the

cellular components results in a material composed of essentially extracellular matrix proteins that can serve as an intrinsic template for cell attachment. Examples of acellularization techniques are freeze-drying (38), treatment with trypsin/EDTA (39-41), detergent treatment (11,41-44), and multi-step enzymatic procedures (45). The use of trypsin has shown to render incomplete acellularization and structural alterations of the matrix (41), although others have reported the complete absence of cellular components and maintenance of matrix integrity (40). Detergent treatment results generally in complete acellularization and preservation of the matrix (41,44). Discrepancies in the outcome using various acellularization techniques are most probably due to proteases present in the native tissue, as their activation leads to the autolysis of extracellular matrix proteins, resulting in damage to the structure and function of the matrix scaffold. Therefore, suitable protease inhibitors should be used (43). To remove any residual DNA and RNA from the matrix, nuclease digestion steps are desirable (11,42,43). The maintenance of mechanical properties depends on the acellularization method used (46) and on the degree of crosslinking, which stabilizes the collagen structure but decreases the ability of tissue ingrowth. In general, non-fixed acellularized valve leaflets have been shown to promote remodeling of the prosthesis by neovascularization and recellularization by the host, as demonstrated in dogs (47) and sheep (48,49), and to possess sufficient mechanical integrity to withstand physiological conditions after implantation, even in the aortic position. The amount of cellular repopulation has been shown to differ among studies, and is thought to depend on the source of the matrix as well as the acellularization technique used (40). However, colonization of the matrices with valvular interstitial-like cells has been reported in all of these studies. Endothelialization - preservation of the subendothelial cellular and matrix components of the implanted valves - was described in both dogs (47) and sheep (40). This endothelialization, as well as the growth, repair and remodeling capability, might be optimized further by seeding cells onto the matrices beforehand, according to the concept of tissue engineering.

All studies using acellular matrices seeded with endothelial cells, showed a confluent layer covering the leaflets when cultured in vitro (38,42,44) and after implantation (39). Acellularized aortic valve leaflets, when seeded with their original valvular interstitial cells, demonstrated all phenotypes present in the native valve leaflet after in-vitro culturing, indicating the potential of cultured valvular interstitial cells to differentiate into various phenotypes (11). The cellularity of the leaflets did not reach physiological levels after in-vitro culturing, either statically (38,39) or dynamically in a bioreactor, in which the opening and

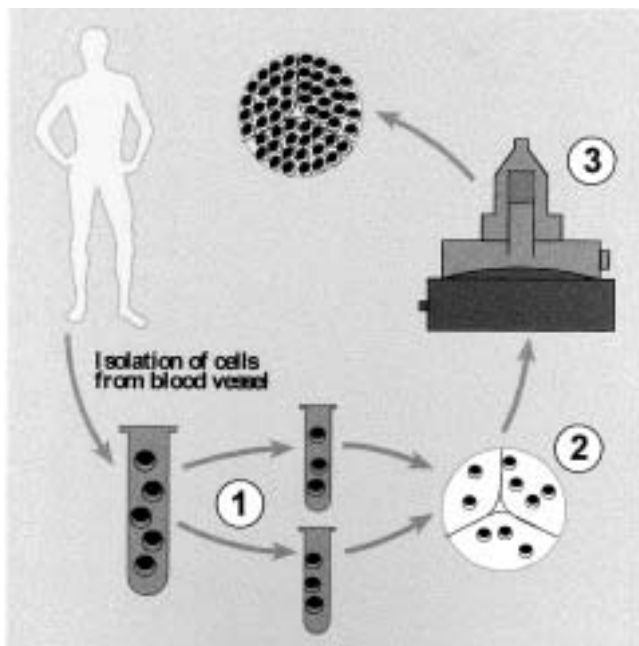


Figure 2: Tissue engineering of heart valves: 1) Isolation of cells from a blood vessel of the patient and separation of myofibroblasts from endothelial cells. 2) Seeding of myofibroblasts onto a scaffold material in the shape of a trileaflet heart valve and subsequent seeding of endothelial cells onto the surfaces. 3) The cell/scaffold construct is placed into a bioreactor to stimulate tissue development.

closing of the valve was simulated (45). At three months after implantation, the leaflets were thickened, which might represent excessive extracellular matrix formation and cellular proliferation (39). Further studies are required to examine the cause of this thickening and to exclude the risk of valve failure after implantation. Although the use of acellularized matrices as a scaffold material might be promising for future clinical use, important disadvantages include the infectious risk when using animal-derived materials (as described earlier), as well as immunological complications. In fact, recent first clinical applications of this concept in children resulted in the rapid failure of heart valves due to severe foreign body-type reactions associated with a 75% mortality (50).

To create a heart valve replacement consisting solely of autologous tissue, the scaffold material must degrade while the valve tissue is developing. Collagen is one of the biological materials that show biodegradable properties and can be used as a foam (51), gel or sheet (37), sponge (52), and even as a fiber-based scaffold (53). However, collagen has the disadvantage that it is difficult to obtain from the patient, and nowadays most collagen scaffolds are of animal origin. Due to the slow degradation of collagen, scaffold material will still be present at the moment of implantation of the tissue-engineered construct. This, on the one hand could lead to the risk of zoonoses, and on the other hand to immunological reactions and inflammation.

Fibrin is another biological material which displays good controllable biodegradable properties. As fibrin gels can be produced from the patient's blood to serve as an autologous scaffold, no toxic degradation or inflammatory reactions are expected (54). Three-dimensional structures can be produced by injection-molding of the cell-gel mixture, followed by enzymatic polymerization of fibrinogen. Degradation is controlled by adding aprotinin, a proteinase inhibitor that slows down or can even stop the fibrinolysis (55-57). Immobilization of growth factors in specific areas has also shown to be feasible (58). As a scaffold material, fibrin also has its disadvantages, mainly that the diffusion and washout of substances into the surrounding medium seems to be reduced compared with other porous matrices (57). Fibrin also tends to shrink, and has poor overall mechanical properties (55,56). Fixation of the gel using poly-L-lysine has been shown to prevent the shrinkage, and the inner tension that subsequently arises results in a more pronounced collagen formation and better mechanical properties (57). Besides its potential as a biological scaffold material, fibrin gel can also be used as a cell carrier in porous synthetic scaffolds (59).

Tissue-engineered heart valves based on synthetic scaffolds

Synthetic materials have been used as scaffold materials for the engineering of various types of tissue (37,54,60). Several attempts to create tissue-engineered heart valve leaflets were also based on synthetic scaffolds such as polyglactin, polyglycolic acid (PGA), polylactic acid (PLA), PLGA (a co-polymer of PGA and PLA) and polyhydroxyalkanoates (PHAs).

A high-porosity synthetic material can be obtained by either fabricating a woven or non-woven fiber mesh or using a salt-leaching technique (35,36). Polyglactin, PGA and PLA are members of the aliphatic polyester family, which degrade by cleavage of the polymer chains following hydrolysis of their ester bonds. The resultant monomer is either secreted in the urine or enters the tricarboxylic acid cycle (35). To fabricate scaffolds for heart valve leaflets, combinations of aliphatic polyesters have also been used, including woven polyglactin and non-woven PGA meshes (61-63) and layers of PLGA and non-woven PGA meshes (64-66). The major limitations of aliphatic polyesters are their thickness, initial stiffness and non-pleiability, all of which make the fabrication of a trileaflet heart valve a difficult process.

The PHA family consists of polyesters built up from hydroxyacids, which are produced as intracellular granules by various bacteria (67). To create trileaflet heart valve conduits, polyhydroxyoctanoate (PHO) (68-71) and poly-4-hydroxybutyrate (P4HB) are used (69). These materials possess thermoplastic properties and can therefore be easily molded into any desired shape, as shown by Sodian et al. using stereolithography (72). The general drawback of PHAs is their slow degradation. Combinations of aliphatic polyesters and PHAs have shown promising results (73-75), in particular the use of PGA coated with P4HB, which combines the high porosity of PGA with the thermoplastic properties of P4HB (74,75).

In order to obtain pure cell populations for seeding, a method has been developed based on the use of fluorescence-activated cell sorting (FACS) (61,76). A mixed cell population, isolated from a blood vessel, is labeled with an acetylated low-density lipoprotein marker, which attaches solely to endothelial cells. The endothelial cells can then be easily separated from the other vascular-derived cells, which comprise a mixture of smooth muscle cells, myofibroblasts and fibroblasts, by using FACS. Using a mixed cell population does not result spontaneously in an endothelial lining covering the leaflets and, therefore, the endothelial cells must be seeded sequentially after an initial neo-matrix formation has occurred only at the surface of the cell-polymer constructs (70). The source of the vascular-derived cells, whether from an artery or a vein, has been shown

to influence the eventual tissue-engineered construct. Constructs seeded with venous cells were superior to those seeded with arterial cells with respect to collagen formation and mechanical stability (77). Considering the source of the endothelial cells, no endothelial lining of the construct was observed when cells were obtained from a vein (70). As the vein is the most promising, most easily accessed cell source for clinical use in the future, further studies should be conducted to identify differences between cells from arteries and veins. Culturing the constructs in a dynamic environment, for example generated by a bioreactor, results in more pronounced and organized tissue formation compared with static cultured constructs (74,75).

Future perspectives

The search for the optimal scaffold material will continue, as even the most promising currently available material - a non-woven PGA mesh coated with P4HB - is still not ideal. This material degrades quickly, but in an uncontrollable manner, whereas degradation of the ideal scaffold should occur on demand in order to ensure mechanical stability over time. Moreover, acidic byproducts arise during the degradation process; these are cytotoxic, and consequently the mechanical properties of this scaffold do not meet the load-bearing properties of the native aortic valve. A basic structure which is prepared from a slowly degrading material, preferably matches the collagen fibers in the leaflets, is combined with a faster-degrading material, and provides the cells with a large attachment area, might be a promising concept.

A completely different approach is that of engineering tissues without a scaffold material. Flexible and elastic tissue layer formation can be produced by folding cultured cell layers and then framing them to provide inner tension (78,79). However, the mechanical properties of these tissues have not yet been shown as being sufficient to allow implantation.

Although veins represent the most promising cell source for clinical use in the near future, attention should be focused on the senescence of the cells and their ability to divide, as the number of divisions must be sufficiently large to bulk the tissue into its original size and to allow remodeling once implanted in the body. A future approach might be the use of vascular cells from young donors, as these may be genetically engineered to remove unwanted gene expression patterns, thereby preventing an immune response of the host (80). Another approach might be the use of stem cells, obtained from fetal or adult tissues (3). Very recently, the use of animal (81) and human bone marrow and umbilical cord cells (82,83) was shown to be applicable to cardiovascular tissue engineering. The field of research with regard to stem cells and their dif-

ferentiation pathways is still in its infancy, the major drawback being the immunogenicity of these cells. In theory, this problem could be solved by the use of genetic engineering (84), and this approach is currently in an experimental phase. Hence, for accelerated clinical application, the use of autologous cells appears to be most appropriate.

The tissue-engineered heart valves implanted into animals were all implanted in the pulmonary position, as their mechanical properties did not allow their placement in the aortic position. A more thorough understanding is needed of the relationship between the mechanical and structural characteristics of the native valve and the stimuli (biological and mechanical) that are required to mimic these characteristics *in vitro*. Tissue-engineered valves in animals display a native-analogous structure, caused by remodeling of the valve *in vivo*. It would be preferable to achieve such native-analogous features prior to implantation. As biological stimuli, adding growth factors to the medium - for example, basic fibroblast growth factor and ascorbate (85) - or the incorporation of growth factors into the scaffold - the so-called 'third generation' biomaterials (86) - each help to stimulate collagen production. The effect of mechanical regulators on collagen production should also be further investigated, as both laminar flow (87) and cyclic strains (88) - and in particular large cyclic strains (89) - have been shown to increase the production of collagen, leading to better mechanical properties of the tissue-engineered constructs. Numerical models, which can be used to predict the remodeling of the collagen architecture in tissue engineering applications, might also be valuable in the generation of mechanically improved tissue equivalents (90).

The concept of bioreactors can be improved by using a controllable, self-regulating loop to measure the pressures and forces that the tissue withstands during its development, and subsequently increase the load on the valve to further improve its mechanical properties. Using the bioreactor, it should be possible to monitor the exact culture conditions (such as pH, pO₂, and pCO₂), and these parameters should be directly adjustable to provide an optimal environment. Another approach might be to apply fetal conditions within the bioreactor instead of mimicking the physiological environment of the adult human body.

Clearly, many disciplines are involved within the field of tissue engineering, and only a synergetic scientific effort will lead to optimal progress. Likewise, it is clear that for every facet in this process, further research is still needed. However, in conclusion it can be stated that much progress has been already made, and that the results obtained thus far show great promise with regard to the future clinical application of heart valve tissue engineering.

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