STEM CELL SEEDED COLLAGEN SCAFFOLDS FOR HEART VALVE REPLACEMENT

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The main treatment for valvular disease consists in surgical replacement with mechanical or biological artificial devices; these are excellent mid-term replacements which greatly improve the patient's quality of life. Both have limited life spans and are associated with bleeding risks (mechanical valves), or degeneration and calcification (biological prosthesis).

Constant progress has been made in order to obtain the perfect heart valve substitute. Regenerative medicine aims to create living heart valves which would overcome the shortcomings of current alternatives. An ideal tissue engineered heart valve (TEHV) should encompass several characteristics: good hemodynamics, appropriate valve geometry, high durability, non-immunogenicity, non-inflammatory, non-thrombogenic and non-calcifying. Additionally, for young patients, it should have the ability to grow and adapt with the patient's somatic growth.

According to the accepted paradigm, the two main TEHV components are the template, or scaffold and the cells (7). The scaffold, either biologic or synthetic, should provide an adequate support for cell attachment and an environment for cells which can remodel the scaffold as a response to physiological stimuli. The cells used in TEHV should be easy obtainable, preferably from autologous sources and should have good expandability in vitro. Once seeded within the scaffolds, these will offer the valve viability, growth potential and remodeling capacity.

Evaluation of TEHV in vivo is typically performed in an animal model which resembles the physiologic and hemodynamic human characteristics. The gold standard for heart valve in vivo testing is considered the ovine model. In vivo periodical functional examination and histological assessment of the explants should be assessed at the follow-up end-point.

The objective of our studies was to compare the in vivo behavior of non-seeded porcine acellular pulmonary or aortic valves with those seeded with autologous adipose derived stem cells (ADSCs) implanted in sheep. Valve-derived scaffolds are preferred because of easy accessibility, outstanding hemodynamic properties and the preservation of biological "niches" after decellularization. We also treat acellular scaffolds with penta-galloyl glucose (PGG) for tissue stabilization, to prevent premature scaffold degradation and to reduce calcification. We chose ADSCs because they are easy to obtain in large numbers and because of their plasticity and ability to differentiate into valve cells. The expectation for the stem cells was to differentiate into interstitial valvular cells in vivo and to provide viability and matrix remodeling before extracellular matrix deterioration.

Porcine aortic roots were decellularized with detergents and enzymes using a pressurized perfusion system and then stabilized with polyphenols. Pulmonary roots were dissected and decellularized by immersion in glass containers on an orbital shaker, also stabilized with 0.1% penta galloyl glucose (PGG), followed by a rapid glutaraldehyde treatment and complete neutralization with glycine to stabilize collagen.

For histology, samples collected from fresh and decellularized pulmonary and aortic cusps and root tissues were fixed in 10% formalin, embedded in paraffin, sectioned at 5 µm and stained with DAPI for nuclei and Hematoxylin & Eosin (H&E) for general structure. DNA was extracted and purified from tissue samples (n=5) using the DN-easy Blood & Tissue Kit (Qiagen) before analysis by Ethidium Bromide agarose gel electrophoresis.

Macroscopically, the decellularized aortic roots appeared intact compared to native tissues. Notably, acellular roots acquired a visible discoloration. Cusp, muscle, sinus, and wall tissue samples, collected after decellularization were analyzed for presence of cells by histology and DNA analysis and compared to fresh(native) tissues. Histology using DAPI nuclear staining showed that all components of aortic or pulmonary roots were fully decellularized. These results were confirmed by H&E staining which was adequate for assessment of overall tissue morphology, presence of cell nuclei, and visualization of the ‘‘pores’’ created by cell removal. DNA analysis by EthBr agarose gel electrophoresis validated the histology data. These results were also confirmed by NanoDrop quantification.

Three weeks before the scheduled implantation, we collected about 5 cm3 of inter-scapular adipose tissue from each animal in sterile conditions and isolated ADSCs using a published collagenase-based procedure. The plasticity of sheep ADSCs was confirmed using differentiation kits (PromoCell, Inc). Cells were propagated for up to 6 passages in the cell culture laboratory and extreme care was exercised to record the source of cells for later autologous implantation. Two days before the scheduled implantation day, the acellular valves were seeded internally with autologous ADSCs by injection of 8-10 million ADSCs in 200 ul PBS into each cusp using a 30G needle (Figure 2, F). The cell-seeded construct was immersed in DMEM/FBS medium and incubated at 37oC, 5% CO2, 90% humidity before implantation.

All animal procedures were performed in accordance to the “Guide for the care and use of laboratory animals”, published by the US NIH (NIH Publication No. 85-23, revised 1996) under a Animal Use Protocol approved by the University of Medicine and Pharmacy Targu Mures Ethical Committee (No.8/04.02.2012). Animals were placed under general anesthesia (oro-tracheal intubation and mechanical ventilation), EKG, invasive arterial pressure, central temperature and puls-oximetry monitoring, with central and peripheral venous cannulation. A left thoracotomy trough the 3rd intercostal space provided an optimal exposure of the right ventricle outflow tract (RVOT) and pulmonary artery (PA). After systemic heparinization and lateral clamping of the RVOT, the proximal end of the conduit was anastomosed to the right ventricle on the beating heart using a 4.0 Prolene Surjet suture; then the distal end was sutured onto the pulmonary artery in the same manner. After the complete ligation of the native pulmonary artery and unclamping of the RVOT, the conduit placed between the RVOT and the PA became a bypass route for the physiological pulmonary blood flow. Postoperatively antalgic, antibiotic, and diuretics were administered for 5 days. Anticoagulant therapy was maintained for the first 30 days and anti-agregant treatment for the entire length of the study.

Monthly transthoracic echography was performed under mild sedation, monitoring the valve hemodynamics, leaflet mobility and thickness, and evidence for right heart remodeling. Animals were randomly divided into three groups: Group A (n=6) consisting of animals with implanted decellularized pulmonary xenografts, Group B (n=6) with implanted decellularized pulmonary xenografts seeded internally with autologous ADSCs and Group C (n=7) with implanted decellularized aortic xenografts seeded internally with autologous ADSCs. Animals were followed for up to 6 months. Explanted valves were analyzed by macroscopic evaluation and histology.

The early (up to 4 weeks) post-operatory evolution of all animals was favorable, with rapid recovery and without immediate complications or early deaths.

All animals (6/6) in Group A survived to 6 months and had physiologic growth without signs of pulmonary or heart failure. Echocardiographic examination revealed valve leaflets with a preserved structure, no impaired mobility and minimal regurgitation, and no inflammation or calcification.

By comparison, animals in Group B and C had a different evolution. After about 1 month of follow-up, animals started to present symptoms of right ventricular failure, including dyspnea, pleurisy and ascites. These symptoms were correlated with echographic aspects consisting of cusp thickening and reduced mobility, resulting in valvular insufficiency and right ventricular dilatation.

Upon macroscopic evaluation, fibrous tissue overgrowth was observed in all valves, covering both distal and proximal anastomoses, the pericardial segments and also slowly enclosing the cusps.

Histology revealed that all valves explanted from Group A presented preserved leaflets integrity. In Group B and C valves, the most notable finding was the presence of a 100-300 um thick, well developed fibrous tissue, rich in fibroblasts and connective tissue covering all surfaces. In most samples analyzed, the cusp tissues were still intact, but covered in fibrous tissue which limited their mobility. No cells of any kind could be detected in any of the cusp tissue, including lack of any seeded cells, infiltrated inflammatory cells or fibroblasts. In addition, no thrombosis occurred at the cusp surface. Calcification was absent from all cusp tissues analyzed.

One important aspect which needs further investigation is to find out whether seeded cells actually survived within the valve interstitium, and whether they were not affected by the mechanical stress to which cusps are subjected each cardiac cycle. This includes bending, stretching, and extending forces which could have affected cell viability. It is possible that stem cells are vulnerable to mechanical stress and thus mechanical adaptation and pre-conditioning in vitro before implantation could be needed. In vitro experiments with seeded valves tested in bioreactors are under way in our lab. In addition, a series of experiments should be conducted with fluorescently labeled cells to evaluate their fate after implantation.

Further improvements in cell seeding procedures are also needed, replacing the single injection with methods capable of homogeneously distributing cells within the valve interstitium. Also, orthotopic implantation of valves would be the next animal model to test, since it will provide additional clinical significance. In this respect, since sheep have a tendency to fibrose over valvular implants, alternative large animal models would need to be evaluated.

Another study was to test the hypothesis that stem cells, as a response to valve-specific extracellular matrix “niches” and mechanical stimuli, would differentiate into valvular interstitial cells (VICs). Porcine aortic root scaffolds were prepared by decellularization.

After verifying that roots exhibited adequate hemodynamics *in vitro*, we seeded human adipose-derived stem cells (hADSCs) within the interstitium of the cusps and subjected the valves to *in vitro* pulsatile bioreactor testing in pulmonary pressures and flow conditions. As controls we incubated cell-seeded valves in a rotator device which allowed fluid to flow through the valves ensuring gas and nutrient exchange without subjecting the cusps to significant stress. After 24 days of conditioning, valves were analyzed for cell phenotype using immunohistochemistry for vimentin, alpha-smooth muscle cell actin (SMA) and prolyl-hydroxylase (PHA). Fresh native valves were used as immunohistochemistry controls.

Analysis of bioreactor-conditioned valves showed that almost all seeded cells had died and large islands of cell debris were found within each cusp. Remnants of cells were positive for vimentin. Cell seeded controls, which were only rotated slowly to ensure gas and nutrient exchange, maintained about 50% of cells alive; these cells were positive for vimentin and negative for alpha-SMA and PHA, similar to native VICs. These results highlight for the first time the extreme vulnerability of hADSCs to valve-specific mechanical forces and also suggest that careful, progressive mechanical adaptation to valve-specific forces might encouragestem cell differentiation towards the VIC phenotype.

As conclusion, acellular stabilized pulmonary roots are excellent valve replacement alternatives. They exhibit adequate hemodynamics and the matrix scaffold is non-immunogenic, non-thrombogenic and non-calcifying in an animal model which typically induces severe degeneration of tissue valves. Notably, cell removal creates pores which could serve as sites for re-cellularization.

Seeding these scaffolds with autologous ADSCs was not conducive to tissue regeneration and at least in the current sheep model, the presence of stem cells was associated with extensive fi-brotic reactions from the host. Further basic and pre-clinical studies are needed to fully harness the potential of stem cells in heart valve tissue engineering.