Remodeling of ECM Patch into

Functional Myocardium in an Ovine Model: A Pilot Study

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ABSTRACT

Background: Previous studies have demonstrated that surgical patches comprised of small intestinal submucosa-derived extracellular matrix (ECM) have biological remodeling potential. This pilot study investigated histological, mechanical, and bioelectrical properties of an ECM patch implanted in the ovine right-ventricular outflow tract (RVOT).

Materials and Methods: ECM patches (2x2 cm) were implanted in four Western Range sheep (wether males, 37-49 kg, age less than 1 year) and explanted at 5 months (n=2) and 8 months (n=2). *In vivo* analysis included epicardial echocardiography and contact electrical mapping. Optical mapping was used to map electrical activity of 2 hearts on a Langendorff preparation. Mechanical testing quantified stiffness. Histological stains characterized structure, neovascularization, and calcification; immunohistochemistry (IHC) assessed cell phenotype.

Results: *In vivo* analysis showed that ECM patch tissue was contractile by M-mode and 2-dimensional echocardiographic evaluation. *In vivo* electrical mapping, and optical mapping confirmed that ECM conducted an organized electrical signal. Mechanical testing of native and ECM patched RVOT tissue showed an elastic modulus of the implanted patch comparable to native tissue stiffness.

Conclusions: At 5 and 8 months, the ECM had undergone extracellular matrix remodeling and neovascularization without calcification. The ECM was populated with locally aligned muscle cells positive for sarcomeric alpha-actinin, CD45, and troponin I and T. In sheep, the ECM patch appears to have the potential of remodeling to resemble native, functional ventricular tissue as evidenced by histological, mechanical, and electrical properties.

Keywords: myocardium, remodeling, angiogenesis

Introduction

Congenital cardiovascular defects remain the leading cause of infant death from congenital malformations.¹ To repair these defects, congenital heart surgeons use many different materials to close defects, create tunnels, or widen passageways. Currently, no ideal patch material exists. The materials available for use, including polyethylene terephthalate (PET; Dacron®), expanded polytetrafluoroethylene (ePTFE; GORE-TEX®) and membrane materials such as bovine pericardium, are associated with significant morbidity. These materials can produce a significant inflammatory response leading to calcification and contraction, and they can become infected or thrombogenic. They are not chemotactic and cannot be functionally degraded, and thus cannot grow with the patient, often necessitating reoperation later in life.² Even native materials such as autologous pericardium, though they do not trigger an immune response, become fibrotic and retracted and can become aneurysmal and arrhythmogenic.^{3, 4} A clinical need exists for a bioresorbable biomaterial for use in cardiac repair applications.

One approach to this problem is the use of decellularized extracellular matrix scaffolds.⁵ In contrast to synthetic biodegradable materials, decellularized scaffolds contain extracellular matrix that can remodel, signal surrounding cells, and immediately serve as a load-bearing scaffold.⁶ Extracellular matrix derived from porcine small-intestinal submucosa (SIS) in particular has been shown to be chemotactic,⁷ and once implanted can become populated with progenitor cells from adjacent tissues as well as marrow-derived stem cells from the circulation.⁸⁻⁹ Following migration and attachment, these cells can undergo proliferation, differentiation, and phenotypic maturation, remodeling the implanted scaffold into functional tissue in the process.^{5,}¹⁰ Furthermore, because all of the porcine cells are removed from the scaffold during processing,

and because collagen is the primary matrix element remaining, these materials are considered minimally antigenic.

In both animals and humans, decellularized, SIS-derived scaffolds have been shown to support vascularization, tissue development, and restoration of regional mechanical functionality in numerous tissues.¹¹⁻¹³ These materials are currently being used in humans for musculotendinous replacement, bladder reconstruction, dura mater replacement, and body wall repair. A commercial SIS extracellular matrix product has also been cleared in the US and Europe for human cardiac tissue repair and pericardial closure as a patch only with no remodeling indication in the US.¹³ In preclinical models, this material has been shown to be repopulated with myocytes¹⁴ and to confer regional mechanical benefit in canine and bovine cardiac-repair models, which has led to its CE approval for cardiac tissue repair in Europe.¹⁵

The ovine preclinical model has been consistently recommended as the most relevant to human cardiovascular performance, demonstrating similar histopathologic and histochemical changes in allograft heart valves compared to allografts in humans.¹⁶ In particular, the juvenile sheep is the preferred model to test the propensity for calcification of allograft material. Prior studies have also demonstrated its appropriateness for studying cardiovascular applications of extracellular matrix scaffolds.¹⁷ The present study assessed the suitability of the juvenile ovine model for evaluation of the histologic, mechanical, and electrical properties of a commercially available extracellular matrix patch following implantation in the right-ventricular outflow tract (RVOT).

Methods

The study protocol was approved by the IACUC at Baylor College of Medicine. All animals received humane care in compliance with “The Guide for the Care and Use of Laboratory Animals,” published by the US National Institutes of Health. Four Western Range sheep (wether males, 37-49 kg, age less than 1 year) were used. Female sheep were avoided due to their higher risk of Q fever transmission. Animals were anesthetized with atropine (0.04-0.8 mg/kg IV), xylazine (0.01-0.33 mg/kg IV), and ketamine (5-20 mg/kg IV) prior to surgery. Cephazolin (15-30 mg/kg IV) prophylaxis was given. A left thoracotomy was performed and a scalpel was used to create a 2-cm long, full-thickness defect in the right ventricle wall, isolated using a side-biting clamp. A 2 x 2 cm decellularized extracellular matrix (ECM) patch (CorMatrix ECM[®], CorMatrix Cardiovascular Inc, Atlanta, GA) was sewn to the defect using a running 4-0 polydioxanone suture, occupying almost the entire anterior wall of the RVOT. After surgery, animal discomfort was managed with a bupivacaine intercostal catheter (2 mL Q 4 hours), a fentanyl patch (1 mcg/kg), buprenorphine (0.01 mg/kg IV q 6 hours), and banamine (1 mg/kg IV q 8 hours) for the first 24 hours. Animals were cared for at Baylor College of Medicine’s Animal Research Facility. Two sheep were randomly selected for reoperation and euthanasia after 5 months, and the other two at 8 months.

Following repeat thoracotomy under anesthesia with glycopyrrolate (0.01-0.025 mg/kg SQ), epicardial echocardiography was performed on each sheep using a Vivid Q portable echocardiography machine (General Electric, Milwaukee, WI). The RVOT and surrounding structures were visualized and M mode and 2-dimensional echocardiographic evaluation was performed by a pediatric cardiologist sub-specialized in echocardiography (author W.V.).

Contact electrical mapping was then performed using a Polaris DX 5575 6-lead catheter (Boston Scientific, San Jose, CA). The electrical activity of the patch and the surrounding right ventricle were assessed by measuring the electrical potential between the leads inside the patch using Vetronics software (BASi, West Lafayette, IN), and data was then transferred from analog printouts through MATLAB software (MathWorks, Natick, MA) to digitized data. The amplitude of the signal was then calculated by measuring the difference between the maximum and minimum of the measured waveform. This electrophysiological assessment was overseen by a pediatric cardiologist sub-specialized in electrophysiology (author J.K.).

For *ex vivo* whole-heart electrical studies, the ECM patched hearts were explanted and the animals humanely euthanized using a sodium pentobarbital overdose of 0.22 ml/kg at a concentration of 390 mg/ml administered IV. The hearts were infused with cardioplegia, immediately placed on ice, and then subjected within one hour to voltage-sensitive dye mapping¹⁸. Hearts were Langendorff perfused with Tyrodes solution and 20 mM Di-4-ANEPPS. Images were taken at 980Hz through a 560nm dichroic by two cameras with 540nm and 610nm bandpass filters. Images were aligned and the ratio of emissions computed for each pixel. Fast Fourier analysis of each pixel utilizing MATLAB was conducted to isolate primary frequencies present. Pacing occurred at 1 Hz; therefore, frequency at 1 Hz was isolated and visible peaks were seen on the epicardium of the heart. From the Fourier analysis, we calculated the phase of the 1 Hz waveform. From the phase analysis, we converted the phase to a time-shift depolarization, in which the depolarization time of the right ventricular face, and the speed and direction of the depolarization wave across the ECM patch were visualized.^{19, 20}

Following optical mapping, the harvested tissue was dissected, reserving 2 mm-wide sections of patched and native tissue for mechanical testing. Additional 2 mm-wide sections were fixed in 10% neutral buffered formalin and dehydrated for later histologic evaluation.

Mechanical testing was performed to compare the elastic modulus (stiffness) and extensibility of native and patched tissue. The width and thickness of each tissue section (1 section from each patch and 2 from the surrounding myocardium of each heart) was measured in 3 positions and averaged. The ends of each length of tissue were affixed to balsa wood mounts with cyanoacrylate (Loctite 435, Henkel, Rocky Hill, CT). The wooden mounts were clamped into an Enduratec 3200 mechanical tester (Bose Corporation, Eden Prairie, MN) and the sample was extended to a taut length, bearing 0 N load. This “gauge length” of the tissue was recorded and preconditioning was performed using 10 stretch-unstretch cycles of increasing stretch magnitude. The sample was then pulled to tension at a rate of 0.4 mm/sec until reaching a final length of 155% of the original length in order to ensure a greater than 150% extension ratio. Load and deformation values were converted to stress and strain by dividing by the sample cross-sectional area (width x thickness) and gauge length, respectively. The elastic modulus was obtained for each tissue section by calculating the slope of the linear portion of the stress-strain curve and its x-intercept represented the tissue extensibility.

Formalin-fixed samples were embedded in paraffin, cut into 5-micron sections and mounted onto glass slides. Hematoxylin and eosin (H&E), Movat's Pentachrome, and Masson's Trichrome stains were used to examine the structure of the ECM and to localize extracellular matrix components including collagen type I, highly hydrated glycosaminoglycans and proteoglycans,

elastic fibers, and smooth muscle. Alizarin red staining was performed to assess the degree of calcification.

IHC analysis was performed to assess the presence of the leukocyte common antigen CD45 (1:25, ab10558, Abcam, Cambridge, MA) and components of the sarcomere contractile complex: sarcomeric alpha-actinin (1:50, ab7817, Abcam, Cambridge, MA), troponin I (1:50, ab19615, Abcam, Cambridge, MA), and troponin T (1:100, ab33589, Abcam, Cambridge, MA). Slides were rehydrated and antigen retrieval performed in phosphate-buffered saline (PBS) at 80°C for 30 min. Slides were blocked with 10% goat serum buffer (Sigma, St. Louis, MO) then incubated for 1 hour at 37°C with primary antibodies. After rinsing in PBS, biotinylated secondary antibodies were applied for 1 hour at room temperature at a 1:500 dilution. Positive staining was then visualized chromogenically with Vectastain Elite ABC and diaminobenzidine kits (Vector Laboratories, Burlingame, CA). All samples were counterstained with hematoxylin. Elimination of the primary antibody served as a negative control for all markers.

Statistics

Data are presented as mean and standard deviation, with 95% confidence intervals (CI). In cases where there are multiple measurements per sheep the number of measurements are reported.

Due to the small sample size, hypothesis testing was not performed.

The study's sponsors were not involved in the study design, collecting analyzing, and interpreting the data, in writing this report, or in making the decision to submit for publication.

Results

All 4 animals survived the surgical procedures and follow-up period. They were sacrificed at postoperative days 143, 174, 237, and 244.

Epicardial echocardiography showed that the patch contracted with the native myocardium, with thickening of the patch region seen during systole (Fig. 1). Contact electrical mapping showed a coordinated electrical signal across the ECM patch at both 5 and 8 months (Fig. 2).

The amplitude of the signal averaged from the patch at 5 and at 8 months was comparable to the native neighboring ventricular tissue (Table 1).

Langendorff perfusion with subsequent paced beating was successful in one heart from each time point. Optical mapping showed that the electrical signal was conducted across the patch, both at 5 and 8 months. Fourier-transform analysis revealed a peak frequency at 1 Hz in the middle of the patch at both time points, corresponding to the stimulating frequency. Time-shift activation maps showed continuity of electrical signal across the RVOT, including the patch region, at both time points (Fig. 3).

Prior to implantation, the ECM patch material had an elastic modulus of 42.9 ± 11.6 MPa ($n=5$), and a value of 1.28 ± 0.9 MPa at 5 months, and 0.976 ± 0.3 MPa at 8 months (Fig. 4, Table 1). The stiffness of the ECM measured at either 5 or 8 months was comparable to neighboring native right ventricle tissue (Table 1).

Gross visual assessment of the patched hearts at 5 months was suggestive of bioresorption (Fig.

5,A-B). At 8 months, the patch thickness approached that of the native tissue (Fig. 5,C). By 8 months, gross muscle appeared visible inside the suture line (Fig. 5,C-D).

Histological analysis of tissue samples at 5 and 8 months revealed evidence of neovascularization and formation of organized muscle cell islands within the ECM patch (Fig. 6, A, B), with cells seen throughout the patch area (Fig. 6,C). Neovascularization was 3.5 times greater at 5 months compared to 8 months (Fig. 6,D) as measured by number of vessels per area (3.08 ± 1.2 vessels/mm² (n=6) versus 0.88 ± 0.3 vessels/mm² (m=6), respectively). By 8 months, muscle fibers inside the patch stained positive for cardiac muscle markers, such as Troponin I (Fig. 6, E). Muscle fiber organization appeared to have increased at 8 months as compared with 5 months, with the corresponding appearance of fascicle-like structures (Fig. 6,F-H). At 8 months, muscle fiber diameter between patch and native tissue was comparable (12.5 ± 2.6 (n=7) vs. 9.7 ± 2.4 μ m (m=7)). No evidence of calcification was observed at 5 or 8 months, as apparent from the absence of alizarin red staining.

IHC staining with antibodies against sarcomeric alpha actinin, troponin T, and troponin I revealed staining in the patch area (Fig. 6,I, J, K) confirming that the cells demonstrated a cardiac myocyte phenotype. Similar results were obtained with antibodies against CD45 (Fig. 6,L), indicating the hematopoietic origin of these muscle cells. There was no staining in the negative-control slides.

Discussion

The goal of this pilot study was to perform an initial assessment of the juvenile ovine model for a

multi-faceted evaluation of the ability of ECM implanted in the RVOT to remodel into functional myocardium. The histological analysis of explanted tissue showed evidence of progressive, time-dependent tissue remodeling, with neovascularization, muscle-cell island formation, and organization of muscle fibers, with eventual resorption of the patch and formation of fascicles (Fig. 5). Interestingly, vessel density was 3.5 times greater at 5 months compared to 8 months. It is possible that more immature cells possessed greater nutrient requirements, and as they became more differentiated, nutrient demand and therefore blood vessel requirement decreased. More efficient metabolism associated with higher vessel density has been reported necessary for cardiac differentiation of stem cells²¹. In addition, muscle fiber diameter in the patch versus native tissue appeared comparable at 8 months. Prior studies have shown significant differences between right ventricle muscle fiber diameter in hearts under different remodeling conditions, suggesting that muscle fibers in the patch are responding to similar loading conditions as those in the native ventricle.²² The tendency for muscle cells to form islets during remodeling of small intestinal submucosa-derived ECM used to repair right ventricular defects has also been demonstrated. Neovascularization appears critical to this process²³.

IHC staining for sarcomeric alpha-actinin, troponin-I, and troponin-T was consistent with a cardiac muscle phenotype for cells found within the patch (Fig 6, I-L), the borders of which were clearly defined by the suture line, which still surrounded the original 2x2cm area (Fig. 5A). This demonstrates that the edges of the cut myocardium did not reapproximate and exclude the patch since that would have caused the sutures lines to be in close proximity. Importantly, the formation of islands of muscle cells occurred in the interior of the patch, not just at the periphery, despite the very large surface area (2x2cm) of the patch. It has been previously reported that the

maximum distance for cellular ingrowth from surrounding tissue into unseeded matrix scaffolding is approximately 0.5 cm.²⁴ The presence of viable islands of differentiated muscle cells in the ECM patch and positive staining with CD45, a marker of hematopoietic origin, suggests that some of the cells populating the ECM were derived from sources other than local myocardial progenitor cells. Other studies have shown that ECM degradation products are not only chemotactic for cells⁷ but attract c-kit positive cells.⁹ It is therefore possible that the cells found within the borders of the patch are marrow-derived. In the future, it will be important to perform analyses to distinguish graft from host cells, for example by providing genetic proof that cells inside the patch are of host origin.

Also of interest in the histologic analysis was the absence of calcification, even at 8 months. This finding is notable in that synthetic and bioprosthetic materials in juvenile sheep are notoriously prone to calcification, which usually occurs within 4 weeks of implantation.²⁵

Implanted surgical materials, such as Dacron, are known to maintain their high stiffness compared to native tissue over time. In contrast, the mechanical stiffness of the ECM decreased over the course of the study, as measured by Young's modulus. By the 8-month time point, the material behavior of ECM appeared similar to that of the surrounding native tissue, having become less stiff and more extensible. In contrast, dysfunctional remodeling would have increased stiffness and decreased extensibility, such as is seen in heart failure²⁶. The similarity between the patch and native tissue mechanical characteristics demonstrates a significant and functional remodeling process.

The ECM showed unique and favorable bioelectrical properties consistent with significant cellular and functional remodeling. At 5 and 8 months, contact electrical mapping and optical voltage mapping both showed evidence that the implanted ECM patch was capable of transducing coordinated electrical signals, and that the patch contained electrically excitable cells that depolarized at the stimulation frequency. Furthermore, Fourier transform analysis identified the dominant and principal frequency in the middle of the patch to be 1 Hz, which was the frequency at which the heart was being paced. These findings are notable, given that acellular patch materials, such as the ECM patch evaluated here, native pericardium, bovine pericardium, Dacron and ePTFE are electrically inert, and only through population with excitable, connected cells can they transduce a depolarization signal.

Limitations and future directions

A significant limitation of this study is the small sample size, confounded by the fact that only 2 of the 4 hearts were successfully reperfused for the optical mapping experiment. There was also no comparator patch material used. There may also be other processes occurring to explain these experimental results that are difficult to delineate with this small sample size. Due to this small sample size, the findings are mostly observational and suggest the potential for remodeling of the ECM patch. Nevertheless, these preliminary results showing functional electrical activity within the implanted patch are remarkable and, to our knowledge, unprecedented for any patch material currently used for human cardiac repair. In the future, this line of investigation is planned to continue using blinded observers, a larger number of animals, comparator arms (native pericardium as control material, sham surgical controls), a 3D activation propagation map of the RV electrical activity, as well as advanced imaging (i.e. MRI) to document and clarify the

change in viability and functionality of the patch over time.

Conclusion

In summary, the ECM patch, when surgically implanted in the ovine RVOT, appears capable of constructive remodeling into functional myocardium as evidenced by histological, mechanical, and electrical properties. Large scale and more detailed studies are needed to confirm these findings in animal models first then clinically. However, this pilot study does present a possible new and exciting solution for cardiac grafting and tissue repair, especially in the pediatric population.

Disclosure

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Figure Legends

Fig. 1: Epicardial echocardiogram of ECM implanted sheep hearts (still). Note thickening of region of patch (arrowhead) during systole. LV = left ventricle. RV = right ventricle.

Fig 2: In vivo electrical contact mapping of ECM implanted in sheep right ventricle. (Above) Trace at 5 months. (Below) Trace at 8 months.

Fig 3: Optical mapping analysis. The temporal activation of the heart is shown at 5 months (A) and 8 months (B) post-implantation, with similar conduction to that of native tissue along the epicardial layer of the ECM patch. Color scale is identical in both images and shows the difference in temporal activation measured from the apex calculated after Fourier analysis. Circles indicate location of the ECM patch.

Fig 4: Comparison of Young's modulus in SIS-ECM implanted hearts versus native myocardium. Logarithmic scale used to report elastic modulus.

Fig 5: Gross visual assessment of implanted ECM patch material. A) 5-month sample with ruler for size reference. B) 5-month sample with black arrows pointing to perimeter of patch. C) Side view of patch thickness. Arrows point to top and bottom of patch pre (right) and post-implant (left). D) 8-month sample with black arrows point to patch circumference. Gross muscle can be seen inside patch.

Fig 6: Histological analysis of ECM patch implanted in RVOT. Movat's Pentachrome stains (A-D): A) neovascularization in 5-month tissue samples (black arrows). B) muscle-cell islands in 5-month sample (black arrows). C) cell infiltration into center of patch at 5 months (throughout). Neovascularization seen (black arrow). Cells surrounding degrading sutures (inside dotted lines) D) neovascularization (black arrows) and muscle fiber organization at 8 months. E) gross muscle remodeling inside suture lines (dotted lines) at 8 months with Troponin I stain. Masson's Trichrome stain (F-H): F) native ventricle muscle fibers at 8 months. G) patch muscle fibers at 8 months. H) patch muscle fibers demonstrating alignment at 8 months. IHC stains (I-L): I) sarcomeric alpha actinin stain at 8 months. J) Cardiac troponin T stain at 8 months. K) Cardiac troponin I stain at 8 months. L) CD45 stain at 8 months. Black bars: 400 microns.

Table 1: Values for contact electrical mapping signal amplitude and mechanical testing elastic moduli.

	Patch at 5 mo	Native at 5 mo	Patch at 8 mo	Native at 8 mo
Contact electrical mapping signal amplitude	4.89±0.27 n=7	4.96±0.31 n=7	1.54±0.63 n=6	1.34±0.69 n=7
Elastic modulus (MPa)	1.28±0.70 n=2	0.58±0.19 n=2	0.98±0.18 n=2	0.61±0.15 n=2
Sarcomeric alpha actinin	132±21.4 n=16	111±12.3 n=16	97.1±21.1 n=13	95.0±22.6 n=12
CD45	153±17.8 n=10	142.9±14.9 n=11	133±16.0 n=7	124±15.9 n=6
Troponin I	160±26.5 n=5	98.6±23.6 n=5	108±33.2 n=4	138±28.7 n=3
Troponin T	152±13.5 n=4	113±27.4 n=4	131±20.2 n=5	126±24.2 n=6